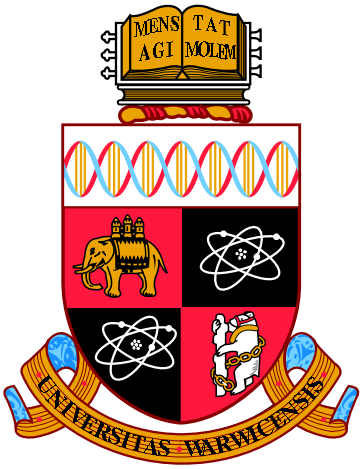

Proteome Adaptations to Phosphate Starvation in *Brassica rapa*

by

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Thesis

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for the degree of

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Declarations

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented (including data generated and data analysis) was carried out by the author.

Abstract

Phosphate is a vital macronutrient yet its availability is often a limiting factor which hinders plant growth. A large proportion of phosphate in soils is organic phosphate (Po) which is inaccessible to plants, it needs to be in its inorganic orthophosphate (Pi) form to be accessible. When grown in Pi deficient conditions plants have adapted numerous phosphate starvation responses (PSR), such as an alteration of their proteome and secreted root exudates, to improve their Pi efficiency. Roots will secrete various compounds such as Purple Acid Phosphatases (PAP) and RNases into the surrounding soil to improve the accessibility of Pi from Po sources for plants to utilise. The majority of research on plants molecular responses to Pi deficiency has been performed on *Arabidopsis thaliana*, with various crop species responses being largely uncharacterised. To gain insight into how the crop species *Brassica rapa* responds to Pi stress they were grown hydroponically and a PSR was induced. The plants root and shoot proteomes were analysed with mass spectrometry to determine proteomic responses to Pi starvation. When Pi starved, the root exudates displayed a significant increase in abundance of various proteins, including secreted PAPs, RNases, and lipid remodelling proteins which could help to improve the accessibility of Pi and improve Pi uptake efficiency. The root and shoot proteomes also displayed an increased abundance of various phosphatases as well as various proteins involved in phosphate transport, lipid remodelling, and signalling. These changes in protein abundance have not been documented before in response to Pi starvation in *B. rapa*, therefore provides valuable insights into which proteins play a role in improving Pi

efficiency in *B. rapa*, providing new and interesting candidates that we could utilise to further improve a plants efficiency in Pi deficient conditions.

Abbreviations

ABC	Ammonium Bicarbonate
ACN	Acetonitrile
APase	Acid Phosphatase
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
BP	Biological Process
BNAP	beta-Naphthyl Acid Phosphate
BSA	Bovine serum albumin
CAA	Chloroacetamide
DAP	Differentially Abundant Protein
DEG	Differentially Expressed Genes
DGDG	Digalactosyldiacylglycerol
DTT	Dithiothreitol
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N‘,N‘-tetraacetic acid

ER	Endoplasmic reticulum
FASP	Filter Aided Sample Preparation
FE	Fold Enrichment
FC	Fold Change
FW	Fresh Weight
GDPD	Glycerophosphodiester phosphodiesterase
GDPDL	Glycerophosphodiester phosphodiesterase like
GO	Gene Ontology
G3P	Glycerol-3-Phosphate
HCD	higher-energy collision dissociation
LC	Liquid Chromatography
LFQ	Label Free Quantification
LR	Lateral root
MAPK	Mitogen-Activated Protein Kinase
MF	Molecular function
MP	Minus Phosphate
MS	Mass spectrometry
MQ	MaxQuant
P	Phosphate
PAP	Purple Acid Phosphatase
PC	Protein Class
PCA	Principle Component Analysis

PEPC	Phosphoenolpyruvate Carboxylase
PEPCK	Phosphoenolpyruvate Carboxylase Kinase
Pi	Inorganic Phosphate
PIC	Protease Inhibitor Cocktail
PM	Plasma Membrane
PMSF	phenylmethylsulfonyl fluoride
pNPP	Para-nitrophenyl phosphate
Po	Organic Ohosphate
PP	Plus Phosphate
PRL	Primary Root Length
PSR	Phosphate Starvation Response
PSI	Phosphate Starvation Induced
P1BS	Phosphate Starvation Response 1-binding sequence
QTL	Quantitative trait loci
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SP	Signal Peptide
SPS	Sucrose Phosphate Synthase
SQDG	Sulfoquinovosyl diacylglycerol
TCEP	Tris(2-carboxyethyl)phosphine
TF	Transcription Factor
TFA	Trifluoroacetic Acid

Chapter 1

Introduction

Plants are essential to providing various necessary materials for food, shelter and medicine. They are sessile organisms, therefore, within a constantly changing environment need to be able to adapt to maximise resources, such as the nutrients available in their immediate area. Over the past century the ever increasing human population has resulted in an increased demand for food and plant materials, but even less land space available in which to grow crops. All these factors lead to a strain on plant growth which means that the supply does not necessarily live up to the demand. Humans therefore need to improve plants growth and development in challenging conditions such as insufficient nutrient availability.

1.1 The model plant *Arabidopsis thaliana*

Arabidopsis thaliana is a useful tool in the world of plant science, often used as a model organism due to its fast reproduction rate, high seed numbers, small size, and a small nuclear genome allowing for quick and efficient analysis [Koornneef and Meinke, 2010]. The scientific benefit to studying it led to an international collaboration (The *Arabidopsis* genome initiative) in 1996 to sequence the genome, identifying 25,498 protein coding genes when completed in 2000 [Kaul et al., 2000]. Work on *Arabidopsis* has since continued from the initial initiative and the updated genome consists of 27,468 protein coding genes which encodes for 39,529 different proteins and splicing variants according to uniprot as of June 2021. The use of *Arabidopsis* in plant research has provided invaluable insights into plant biology, pathology, growth and development. Due to how well studied and characterised *Arabidopsis* has become over the years, it is a useful tool to determine the effects of

differing conditions on the growth and development of the plants in comparison to control conditions. The benefit of a well annotated genome and proteome means that the detection of adaptations in response to different conditions can be interpreted more accurately than in plants that are not so well annotated.

1.2 The Brassica Family

Arabidopsis is a member of the Brassicaceae family. A vast majority of plant research is performed on *Arabidopsis* plants, however *Arabidopsis* is not a crop species and has little economic value. Therefore turning some research focus away from *Arabidopsis* onto *Brassicas* and transferring knowledge from model to crop species, could lead to more practical applications in the field of crop science.

The Brassicaceae family are large diverse plant group that includes the genus *Brassica*, commonly named the cabbage family. The triangle of U represents the relationship between the members of the *Brassica* family (Fig. 1.1) [N. Nagaharu, 1935]. The classification of the members of the *Brassica* family are based on the three genome types that are diploid species, *Brassica rapa* (AA genome), *Brassica nigra* (BB genome) and *Brassica oleracea* (CC genome). Cross breeding these species together formed three other allopolyploid species, *Brassica juncea* (AABB genome), *Brassica napus* (AACC genome) and *Brassica carinata* (BBCC genome). *Brassica* plants are cultivated globally as major crop species for food and oilseed, and their global use makes *Brassica* crops an important species to study and understand.

6.1 million hectares in the UK is arable land used for crops, which is 25% of total land area. In 2018 583,000 hectares was used for *B. napus*, also known as oilseed rape, making it the 3rd largest land use area for crop growth in the UK, after wheat and barley (Agriculture in the UK, 2018), which means *Brassicas* are a highly important crop family in the UK [Department for Environment, Food and Rural Affairs, 2018].

Brassica and *Arabidopsis* genomes are closely related and have many shared homologues. But *Brassicas* have been less researched at the molecular level due to it being harder to work with than *Arabidopsis*. The full genome sequence of *Brassica* was slower to come about, and genetically modifying the plants is more difficult than in *Arabidopsis*. Generating transgenic lines for *Arabidopsis* is much easier, with large catalogues of seed stocks available for purchase. Therefore *Arabidopsis* is more commonly studied and offers useful insights to the workings of plants however the

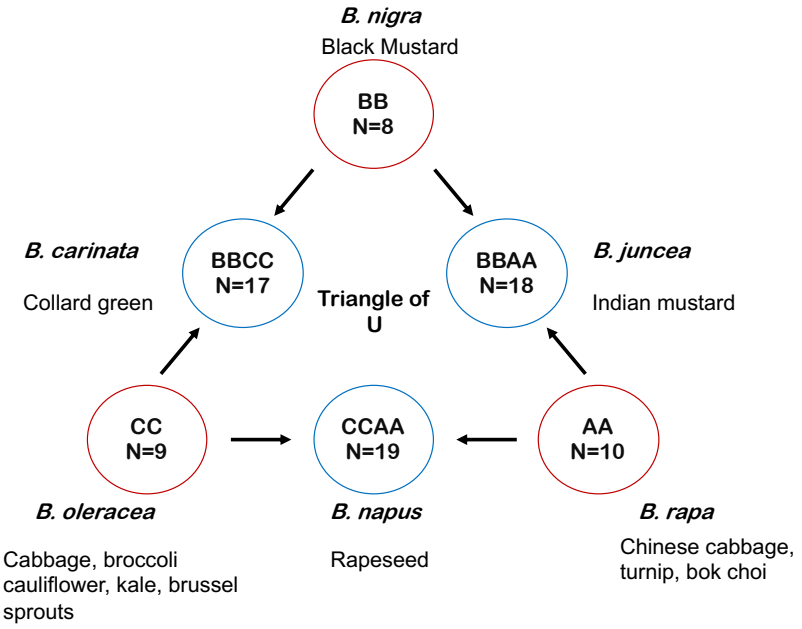

Figure 1.1: Triangle of U: Representing the cabbage family and the relationships between the *Brassica* species. Within the circles indicates the letter that represents its chromosome architecture with the number saying how many chromosomes that species has. Outside the triangle gives some examples of what crops belong to each species.

transfer of knowledge to other plant species is not exact. Therefore, more *Brassica* research is important and offers novel and interesting insights in crop science. *B. rapa* was a major focus in the research displayed in this thesis, and is an important crop species which includes turnip, chinese cabbage and yellow sarson. Another issue with working with *Brassica* is the multiple copies of genes. *B. rapa* is a mesopolyploid species whose genome underwent a genome triplication event thought to have occurred 13-17 million years ago [Wang et al., 2011b]. In some cases for every gene in *Arabidopsis* there are multiple homologues in *B. rapa*, but the *B. rapa* genes maintain a high nucleotide conservation with their *Arabidopsis* homologues with an average 87% sequence identity [Mun et al., 2009, Paterson et al., 2001]. *B. rapa* has 45,985 genes determined by Zhang et al. [2018] with and 47,250 proteins acording to the Brassicaceae Database *B. rapa* version 3.0 [Chen et al., 2022]. This increase in genome size makes for a challenge when unearthing the plants proteome and the functions of the proteins, and specifying which of the homologues are responsible for the activity within the plants.

1.3 Phosphate is an essential nutrient for plants

Plant growth is dependent on light, water, carbon dioxide, and nutrients, requiring macronutrients such as nitrogen (N), potassium (K) and phosphate (P). Phosphorus is vital for plant growth and is involved in many aspects of plant development. It is a key component within plant cells that makes up 9% mass of nucleic acids [Elser, 2012], contributes to the energy of cells in the form of ATP and AMP, is a component of the membrane phospholipid bilayer, and is a component of some enzymes required for photosynthesis [Elser, 2012, Jeng., 2010]. P is also a key player in the growth rate hypothesis which suggests that variability in the P content of living things reflects a growth-related demand for the construction of ribosomal RNA [Elser et al., 2003].

Despite the importance of P to plants, 30-80% of soil P, depending on soil type and previous usage, is unavailable for plants to utilise [Wang and Liu, 2018]. This is because P is readily bound in its organic phosphate (Po) form, forming complexes with metals and organic salts. Po is not accessible for plants to take up so must be hydrolised to inorganic orthophosphate (Pi) to be accessible. The most common Pi in soils are $H_2PO_4^-$, and HPO_4^{2-} [Wang and Liu, 2018]. The availability of Pi is often a major limiting factor hindering plant growth and development.

To combat low Pi availability, farmers will use P fertilisers to improve crop yields. Unfortunately, use of fertilisers is not a sustainable solution to improving crop yield. P fertilisers are developed by mining phosphorus from phosphorus rocks, this is a finite resource, and the estimates of how long it will last varies from 70-200 years if the current rate of use is maintained [Dawson and Hilton, 2011, Lopez-Arredondo and Herrera-Estrella, 2012]. Approximately 18 million tons of phosphorus is mined annually to use as fertilisers, with 5 countries controlling 88% of the worlds phosphate rock [Cordell and White, 2014]. The monopoly on phosphate rock with export tariffs in place has ensured an economic interest in phosphate availability for crop yields [Cordell et al., 2011, Cordell and White, 2011]. Further issues which arise from using fertilisers are the environmental consequences, such as soil degradation and eutrophication of local water sources when the fertilisers get washed away [van Beusekom, 2018]. These issues with fertilisers mean that other ways to improve plant access to P and tolerance of low Pi conditions have to be utilised, rather than just relying on the application of fertilisers to improve crop yields. A more sustainable approach for improving crop yield may be to enhance a plants ability at taking up the available Pi, as plants have adapted numerous responses to improve their Pi

efficiency when in Pi deficient conditions.

1.4 The phosphate signalling pathway

The complete pathway of P signalling during both high and low Pi availability involves multiple other signalling paths, such as iron and zinc signalling, all being interconnected, as well being interconnected with different hormone signalling pathways [Xie et al., 2019]. This means that the complete P signalling web is not yet fully untangled and understood.

Primary root tips are where exogenous Pi deficiency is sensed [Svistoonoff et al., 2007] but the communication of Pi status within the cells through the whole plant requires systemic signalling pathways. The exact Pi sensor is unknown, with some speculation that certain phosphate transporters could play a role in the sensing activity [Zhang et al., 2014], with Pi transporter Pho84 in yeast having been characterised as having phosphate signalling activity [Popova et al., 2010] similar to nitrogen transporters in *Arabidopsis* also having nitrate sensing activity [Ho et al., 2009]. The majority of studies on Pi signalling have been undertaken in yeast and *Arabidopsis*, with other plant species being largely uncharacterised. A complicated root to shoot and further shoot to root communication network are expected to play a role in Pi signalling and maintaining Pi homeostasis.

It has been reported that the transport of certain signalling molecules such as Pi, cytokinins and strigolactone from the roots into the shoots via the xylem act as a message of Pi status, these molecules get "decoded" in the shoots to determine that there is low Pi availability [Lucas et al., 2013], which then triggers a phosphate starvation response (PSR) in the shoots as well as sending shoot to root signalling messages to further induce a PSR in the roots. Long distancing signalling compounds derived from the shoots such as MicroRNs, sucrose, proteins, and other metabolites travel through the phloem from source leaves to sink tissues to regulate P homeostasis (Fig. 1.2) [Chien et al., 2018, Zhang et al., 2014]. Sucrose transport in the phloem and increased concentration is also a signalling system to Pi stress and act to induce PSR [Hammond and White, 2008].

While the roots respond to Pi stress by trying to improve the acquisition of Pi (improved phosphate uptake efficiency), the shoot PSR is to improve the recycling and remobilisation of Pi to maintain Pi homeostasis (Improved phosphate utilisation efficiency)[Zhang et al., 2014].

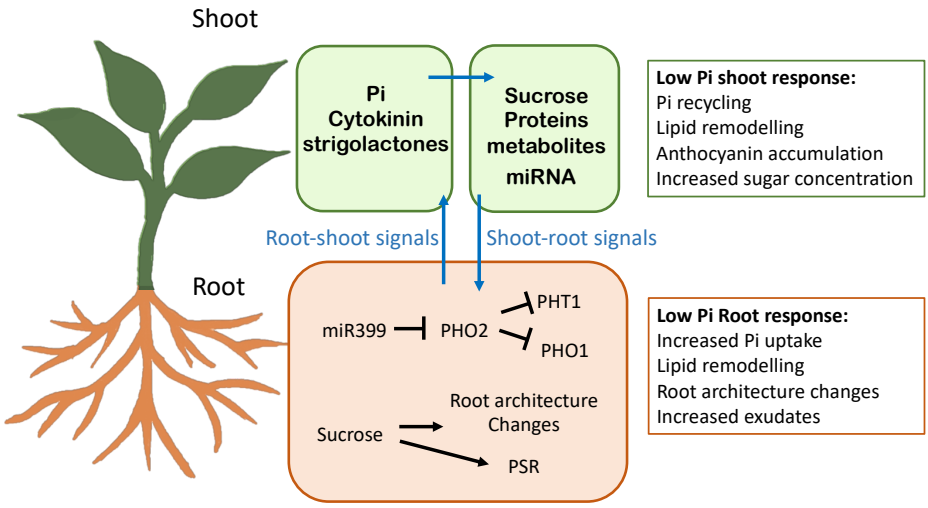

Figure 1.2: Root and shoot communication in Pi signalling: Signalling molecules (Pi, cytokinin, strigolactone) from the root to shoots travel up the xylem, indicating low P availability and in return shoot to root signalling molecules (e.g. microRNA's sucrose, Pi, surgers, proteins and other metabolites),travel via phloem translocation back to the roots and induce a PSR. Shoot PSR results in improving Pi utilisation efficiency, root PSR results in improving Pi uptake efficiency.

Pi concentration within plant cells is between 1-10 mM, which is generally higher than the concentration of Pi found in soils at 1-10 µM [Srivastava et al., 2018]. When there is an ample supply of Pi within cells plants have to prevent further Pi uptake. Extreme Pi within cells can lead to toxicity causing chlorosis and necrosis in leaves, reported in *Arabidopsis* and rice [Chiou et al., 2006, Liu et al., 2010], and increased Pi within leaves can decrease the activation of rubisco and inhibit photosynthesis [Takagi et al., 2020]. Therefore, it is important that a PSR and phosphate starvation induced (PSI) genes are only activated when in low Pi conditions to avoid excessive Pi uptake and accumulation.

The sensing of Pi status in plants is still being researched. SPX domain proteins (SPX1/2) are known to act as a Pi status sensor in *Arabidopsis* and rice and leads to the activation of PSI genes [Puga et al., 2014, Wang et al., 2014b]. The signalling molecule inositol-pyrophosphate (InsP) is reported to be a key player in the activation of PSI response genes alongside SPX1. When there is ample Pi levels then the production of InsP8 is increased. InsP8 binds to SPX1 [Dong et al., 2019] causing it to bind to the transcription factor (TF) PHR1/2 (PHOSPHATE STARVATION RESPONSE 1/2) or its close homologous PHL1/2 (PHR LIKE 1/2) effectively inhibiting the TF from activating the PSI genes under control of the P1BS (Phosphate Starvation Response 1-binding sequence) element in the promoter region of PSI genes [Rubio et al., 2001]. When in low Pi conditions, phosphatases act

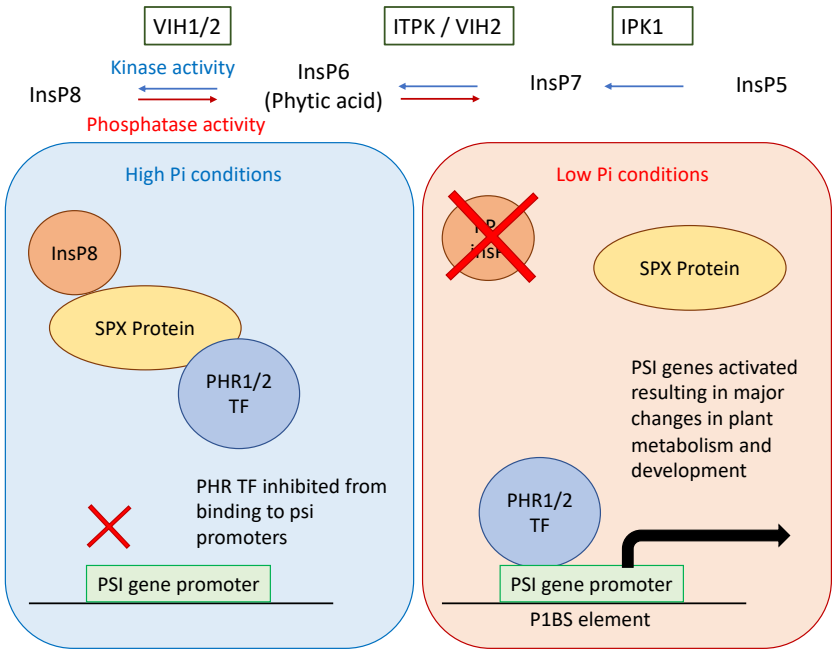

Figure 1.3: Pi sensing and PSI gene activation: When in ample Pi conditions the signalling molecule inositol pyrophosphate InsP8 is synthesized by a variety of kinase enzymes. InsP8 binds to SPX domain protein, this then caused the SPX domain protein to bind to the TF PHR1/1 and PHL1/1 inhibiting them from activating the expression of PSI genes. When in low Pi conditions InsP8 compounds get hydrolysed by phosphatases, breaking them down, resulting in SPX proteins releasing the PHR1/2 TF, which now free to bind to the PSI gene promoters, resulting in PSI gene expression inducing a PSR.

upon the InsP8 molecule breaking it down, and in the absence of InsP8 the SPX proteins release the PHR TF, leaving them free to bind to the PSI gene promoters activating the expression of PSI genes resulting in a PSR being initiated (Fig. 1.3). The biosynthesis of InsP8 is related to the level of Pi within cells, interestingly, some of the enzymes responsible for the biosynthesis of InsP8 via kinase activity in *Arabidopsis* include 2 bifunctional pyrophosphate kinase/phosphatases (VIH1/2), whose activity changes to either synthesize or breakdown InsP8 depending on the level of Pi present [Zhu et al., 2019].

1.5 Phosphate starvation response improve plants Pi efficiency

After low Pi levels have been detected and PSI genes activated, a PSR is induced which works to redirect the plants resources to improve Pi efficiency by improving the plants Pi uptake, use, and utilisation efficiency. Plants have evolved numerous ways to improve Pi uptake and adapt to low Pi conditions [Fujii et al., 2005]. These responses include changes to the root architecture; plants under low Pi conditions

will increase their lateral roots number to enable top soil foraging of Pi (due to the low mobility of Pi it tends to accumulate in the top soil) [Lynch and Brown, 2001]. Increased lateral roots has been observed in multiple plant species, such as lupin, rice, maize and *Arabidopsis*. However different species will sometimes respond differently to Pi levels, for example their primary root length (PRL) response, some species such as *Arabidopsis* and lupin will see a decrease in PRL while others, namely rice and maize will have no change in PRL in response to Pi levels [Castrillo et al., 2017, Gruber et al., 2013, Lambers et al., 2015, López-Bucio et al., 2003, Péret et al., 2014]. The root cells will also induce various biochemical PSRs which lead to improved Pi efficiency.

While in Pi sufficient conditions PHO2, an E2 conjugase, leads to the ubiquitination and degradation of PHT1 phosphate transporters. PHO1 and PHT1;3 transporters translocate phosphate from the root to the xylem for distribution to the rest of the plant, observed in *Arabidopsis* and rice plants [Chang et al., 2019, Mlodzinska and Zboinska, 2016, Poirier et al., 1991]. When in Pi deficient conditions, miR399 synthesized within the shoot tissue gets translocated to root cells [Chiou and Lin, 2011], here it acts to break down the mRNA of PHO2, and hence blocks the ubiquitination of PHT1 and PHO2 transporters, leading to an increased abundance of Pi transporters on the root plasma membrane for improved Pi uptake efficiency during Pi deficient conditions [Bari et al., 2006] (Fig. 1.4). In *Arabidopsis* these transporters are the AtPHT1 family and result in increased phosphate uptake [Puga et al., 2017]. When in deficient Pi conditions stored P in the vacuole will be utilised by the roots and there will be less transport of Pi to the rest of the plant. Deficient Pi conditions will also lead to an increase and altered secretion of various compounds from the roots into the rhizosphere.

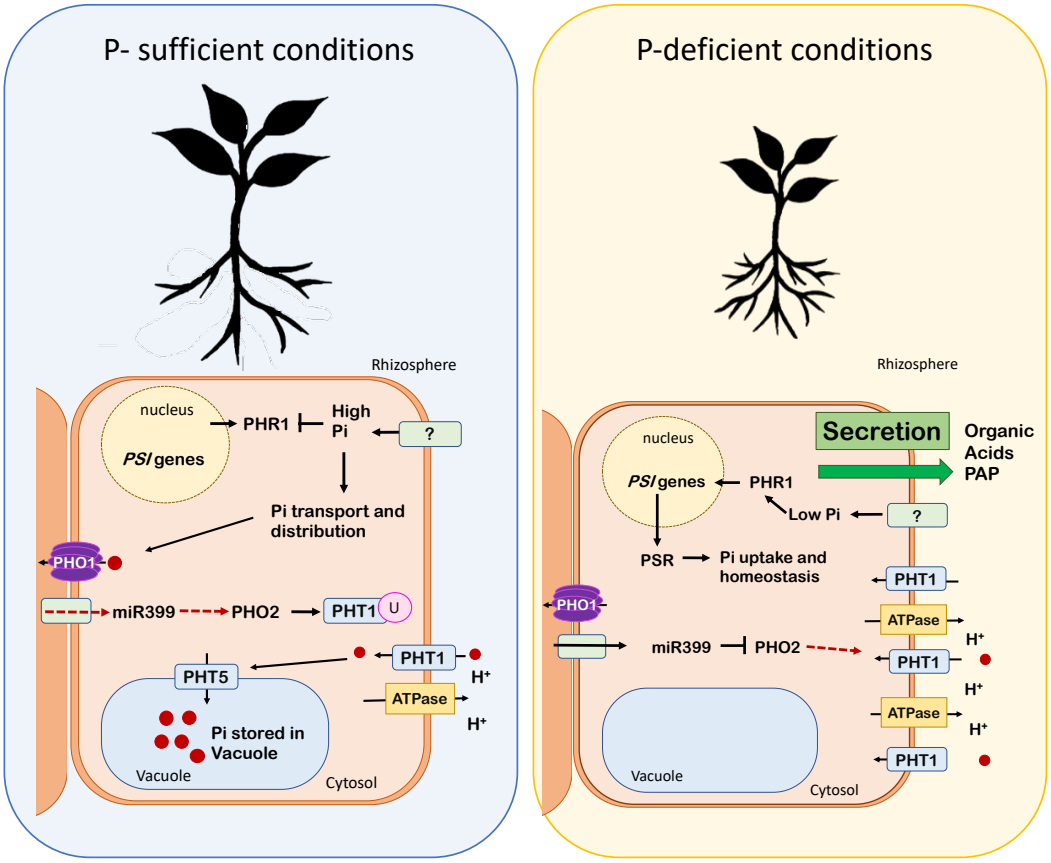

Figure 1.4: Plant root PSR: Different Pi conditions lead to different responses in the roots. In Pi deficient conditions plant growth is inhibited and roots will increase the lateral root number to increase Pi scavenging. In Pi sufficient conditions root cells will detect sufficient Pi so PSI genes remain inactive. PHO2 signals the ubiquitination of phosphate transporters (PHT1), and Pi that gets taken up is distributed to the rest of the plant or stored in the vacuoles. In Pi deficient conditions root cells will detect low Pi and PSI genes are activated leading to a phosphate starvation response for the improved Pi uptake and homeostasis. The micro RNA miR399 gets transported from the shoot to root region and inhibits PHO2 so that PHT1 proteins do not get degraded, leading to increased PHT transporters on the root plasma membrane. Secretion of various compounds into the rhizosphere is increased to improve Pi accessibility and uptake. Stored Pi is utilised. Arrows represent pathway steps activated, red dotted lines are pathway steps that have been blocked, and blocked arrows indicate inhibition steps of a pathway. Red circles represent P.

1.5.1 Root secretion as a phosphate starvation response

Plant roots will secrete various compounds, such as proteins, organic acids, amino acids, sugars, anions and phenolic acids, into the surrounding soil to help utilise reserves from their environment. The types of compounds secreated by the roots can change to adapt in response to various stress conditions. Secreted compounds play a variety of roles in helping to shape the rhizosphere into a beneficial environment for the plants to thrive in, such as encouraging the colonisation of beneficial microbes, defending against pathogenic microbes, as well as improving the availability of nutrients (Fig. 1.5) [Canarini et al., 2019, Haichar et al., 2014].

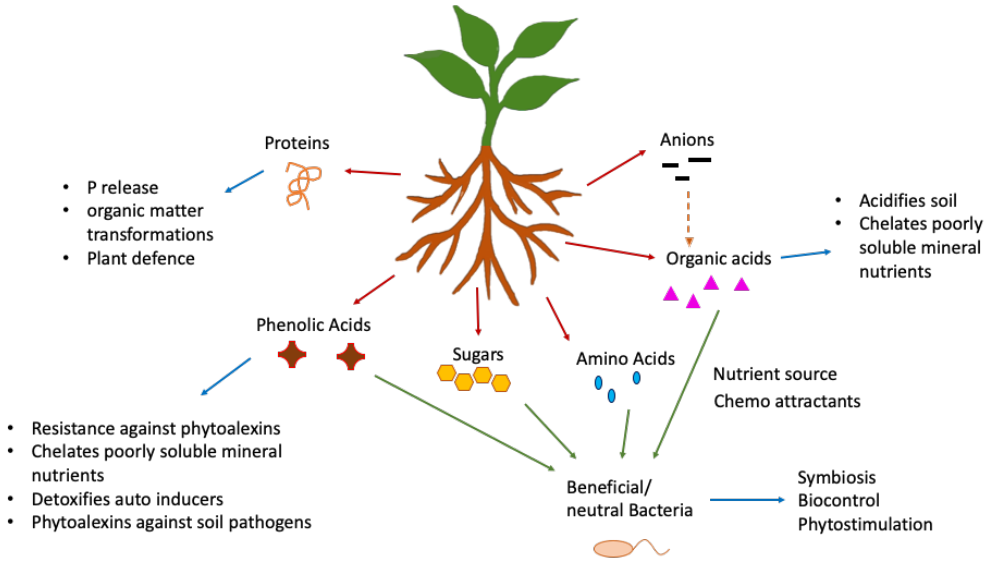

Figure 1.5: Plant root secretion: Roots secrete a variety of compounds into the surrounding rhizosphere which play varying roles. Red arrows indicate compounds secreted from the plant roots, green arrows indicate the compounds which help to promote mutualistic, beneficial or neutral bacteria for colonisation. Blue arrows lead to explanations of what some of the compounds functions are and how they help the plants growth and health. The orange dotted arrow indicates that the anions role is to help improve the secretion of organic acids into the rhizosphere.

Root secretions are altered under nutrient stress conditions in order to improve the plant's ability to access nutrients. The secretion of proteins such as acid phosphatases and RNase enzymes [Abel et al., 2000, Raghothama and Karthikeyan, 2005] into the surrounding soil have been documented to improve P mobility and increase Pi availability for uptake in rice and *Arabidopsis* [Bozzo et al., 2002, Lu et al., 2016, Wang and Liu, 2018]. Secreting carbon compounds and organic acids such as citrate, malate, or oxalate promotes the growth of microbes in the rhizosphere in a mutually beneficial relationship, and can also help to solubilise phosphate so that it is more amenable for uptake by plants, some metabolites such as 3-hydroxypropionic acid have been shown to improve the solubilization of phosphate therefore improving its accessibility [Pantigoso et al., 2020, Zhou et al., 2015].

Many plant species will form mycorrhizal associations with fungi within the rhizosphere in a mutualistic symbiosis. This relationship means the fungi help the plants to absorb nutrients and in return the plants provide photosynthetic carbohydrates [López et al., 2008]. An example of mycorrhizal interactions is when maize plants will form mycorrhizal associations with arbuscular mycorrhizal fungi which results in increased Pi uptake and improved plant growth [Real-Santillán et al., 2019]. In 2006 a comprehensive review by Wang and Qiu covered a survey of 3,617 land plant species in 262 plant families and found that 92% of families form mycorrhizal associ-

ations, but that the Brassicaceae plant family are unable to form these associations [Wang and Qiu, 2006]. Despite the lack of association, *Brassica* plants are still able to benefit from microbes within the soil in a mutually beneficial relationship, but through the less direct secretion of compounds into the surrounding area.

1.6 Phosphatase proteins play a role in plants phosphate starvation response

Phosphatase proteins can improve the availability of Pi from Po sources, by catalyzing the release of Pi (Fig. 1.6). A group of phosphatase proteins called acid phosphatases are able to be secreted from plant roots into the surrounding environment. A subgroup which are commonly responsive to Pi deficiency are the Purple Acid Phosphatase (PAP) family, so named as they appear purple in solution. Some of these PAP proteins can get secreted from the roots directly into the surrounding environment in response to low Pi conditions, this has been observed in *Arabidopsis* and rice plants, and results in the increased availability of Pi in the rhizosphere for the plants to acquire [Lu et al., 2016, Sun et al., 2018, Wang and Liu, 2017].

Some PAPs are root bound and are active extracellularly on the surface of the roots in the environment to improve the Pi availability and uptake from the rhizosphere [Tian et al., 2012, Zhang et al., 2011]. Some studies on PAP proteins include some successful use of PAP overexpression lines in *Arabidopsis*, which results in improved uptake of Pi compared to wild type lines [Tian et al., 2012], indicating improved Pi efficiency of the plants due to the increased abundance of PAPs. Therefore PAPs are a interesting target in plant PSR to improve plants Pi efficiency.

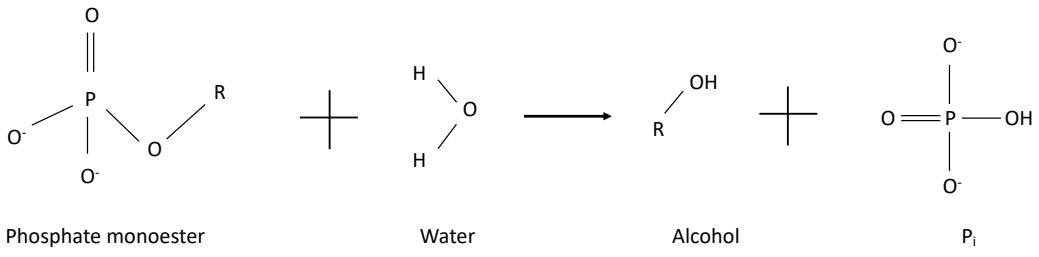

Figure 1.6: Phosphatase reaction: The catalytic activity of a phosphatase is to hydrolyze a phosphate ester resulting in the release of Pi and an alcohol.

1.7 Phosphate transporters increase in abundance during phosphate starvation in plants

As the name suggests, phosphate transporters play an important role in Pi acquisition, they are required not only to take up Pi from the environment but are also needed to redistribute Pi to the rest of the plant and to various cell compartments, such as the vacuole for storage.

Under sufficient Pi conditions, there are a variety of Pi transporters that are utilised, and the most well studied family is the Phosphate Transporter (PHT) family which consists of members PHT1-5, which have been well categorised within *Arabidopsis*, but less so in *Brassica*. The PHT1 family are plasma membrane influx transporters, that import Pi from the surrounding environment into the roots. PHT transporters are proton coupled transporters, therefore function by importing protons along with Pi [Nussaume et al., 2011].

Once within the roots the Pi can be stored within the vacuole or get transported to the xylem for distribution to the rest of the plant via the PHO transporters. The other PHT proteins; PHT2, PHT3, PHT4, and PHT5 transport Pi into the chloroplast, mitochondria, golgi/chloroplast, and vacuole respectively (Fig. 1.7) [Mlodzinska and Zboinska, 2016]. Other transporters not within the PHT family such as the SPX-MFS3 vacuolar phosphate transporter are also involved in the efflux of Pi that is stored in vacuole to redistribute to cells when needed [Srivastava et al., 2018].

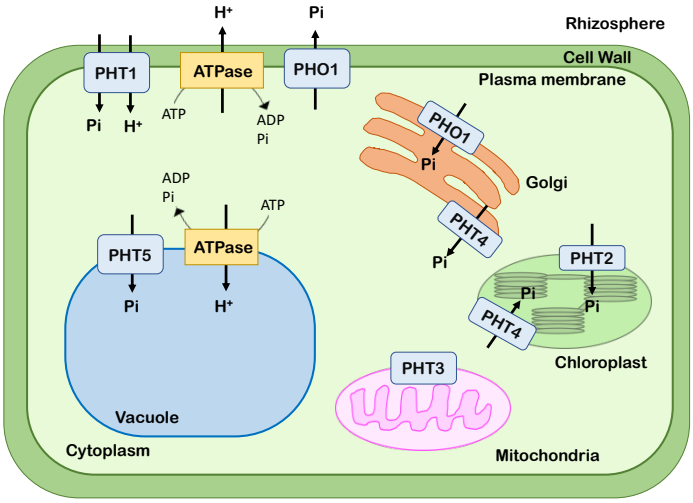

Figure 1.7: Plant phosphate transporters: PHT family of transporters annotated from *Arabidopsis* proteins with the different localizations which distinguish the different members of the PHT family.

The increase in abundance of different PHT1 proteins is a part of various plants PSR to improve Pi uptake. *Arabidopsis* high affinity Pi transporters PHT1;8/9 are induced during Pi starvation to improve Pi acquisition [Remy et al., 2012]. The rice PHT1;3 is also increased in abundance during Pi starvation [Chang et al., 2019], indicating similar responses of Pi transporters to Pi starvation in different plant species.

1.8 Proteomic techniques

1.8.1 Mass Spectrometry

Mass spectrometry (MS) can be used to identify proteins present within a complex mixture. MS can also be used to identify modifications of proteins, such as phosphorylation, methylation and carbonylation of proteins which can be an important tool in mapping protein signalling pathways.

Before the proteins can be identified they first need to be digested into peptides, in this thesis all proteomic experiments used trypsin for digestion, which cleaves proteins at the C-terminal of arginine and lysine residues [Olsen et al., 2004]. Because an average protein yields 20-50 peptides, tryptic digest results in a very complex mixture which needs to be separated using reverse phase liquid chromatography (LC) column. The LC columns are made up of C_{18} coated silica particles, and peptides which have more hydrophobic capacity bind more strongly to the column than those with more hydrophilicity [Shire, 2015]. The peptides are eluted on a gradient of organic solvent over time. This use of the column helps to spread out the input of peptides into the MS machine which is useful for not overloading the machine and allows greater coverage of the peptide samples to be identified.

Once the peptides exit the LC column they are ionised by electrospray ionisation to give them a positive charge and enter the MS machine [Ho et al., 2003]. In this thesis all proteomic experiments were performed on an Orbitrap Fusion Thermo fisher instrument. Once in the instrument an electric field is used to guide charged particles through it, any neutral or negatively charged particles will be lost. The charged peptides undergo two MS events, MS1 is the precursor scan and detects the peptide as a whole to give the mass to charge ratio of the peptide, this is performed within the Orbitrap of the MS machine and has a high mass accuracy [Hu et al., 2005]. After the precursor scan, the peptide will be selected to undergo an MS2 scan within the ion trap of the machine. The peptides are fragmented into smaller fragments and the mass to charge ratio of the smaller fragments is measured, the

data for each peptide fragment MSMS scan is fourier transformed and a spectrum is obtained which can be used to sequence the peptide and hence identify the protein present.

Post translational Modifications (PTM) are an essential part of protein functions. Protein phosphorylation is a reversible PTM which is the most common type of PTM, it is the addition of a phosphoryl group to an amino acid residue and can be identified using MS. Three amino acid residues that are commonly phosphorylated and are easily detected by MS are serine, threonine or tyrosine [Park et al., 2012], which can cause the loss or gain or change of function of proteins [Mann and Jensen, 2003], therefore identifying changes in phosphorylation is a useful tool to try and gain insights into changes in proteins and plant responses during stress conditions. In order to determine the phosphorylation of peptides and the different phosphosites, phosphopeptide affinity purification is used, this is a method used to enrich for the peptides which have a phosphorylation modification. One way to perform the enrichment is through metal chelation using TiO_2 beads in which the selective interaction of phosphorylated peptides bidentately bind to TiO_2 [Aryal and Ross, 2010]. The enriched peptides are then run in the MS to be identified with their phospho modifications. The enrichment allows a better coverage for the identification of peptides which may normally be in low abundance so get overlooked in the MS.

1.8.2 Data Analysis

The MS/MS spectra generated by MS are assigned to peptides in a database using pattern matching within MaxQuant (MQ). MQ uses an algorithm called Andromeda that works through probability matching of the generated MS spectra to the predicted peptides within a protein database, then assigns a score for the probability of the match [Cox et al., 2011]. MQ will also deduce Label Free Quantification (LFQ) intensities by using the intensity of the peak coming off the LC column and matching to assigned peptides. The area of this peak is then compared between samples to allow quantitative analysis of proteins identified between samples [Bantscheff et al., 2012].

When analysing the proteins in a sample it is incredibly important that there is a accurate and good quality proteome available so that the spectra can be assigned and matched to predicted peptides. If there is nothing to match the spectra to then it will not be assigned and the peptide will be missing, even one amino acid difference will result in a mass shift and unassigned spectra.

Searching for PTM relies of searching for variable modifications in the predicted peptide database. The spectra taken to match to the peptide must be searched with or without the potential presence of the change in mass shift that is caused from a modification being present, making the search computationally more intense as the database increases in size to account for variable mass shifts. MQ gives a site localisation score to indicate which amino acid residue the modification is on.

After MS/MS spectra has been assigned to a peptide and the precursor peak area calculated to give the LFQ intensity by MQ then statistical analysis is performed in Perseus. Perseus is a software tool used to analyse LFQ proteomic data as well as phosphoproteomic data [Tyanova et al., 2016] allowing straightforward analysis of MQ searches. It allows the quantitative analysis of protein and phosphopeptide abundance between samples by filtering of low confidence proteins, statistical analysis and graphical output of the data.

Once differentially abundant proteins have been identified, analysing the predicted functional changes of these proteins in response to the stress conditions is then also required. Gene ontology (GO) classifies genes and their protein products based on their biological process, molecular function, or cellular compartment. These terms can be used to give a general idea of the function and activity or cellular location of a protein or group of proteins. GO-term enrichment analysis is a good way to determine if there are particular biological processes or molecular functions assigned to identified proteins which are enhanced in a group of differentially abundant proteins (DAP). Enrichment analysis compares the identified proteins GO terms to the total number of protein GO terms which are present within the organisms proteome, to see if the GO-terms are significantly enriched. There are some drawbacks to using GO term analysis, as not all proteins have GO terms assigned to them, and the GO terms are heavily biased towards stress responses. The terms are also hierarchial therefore some very broad terms can be applied to a number of different functions and processes. Therefore getting specific information is not always possible, but getting an overall view of the changes in proteins that occur is a good way of getting a broad idea of the protein functions, but we also have to be aware that the functions are also predicted so our samples wouldn't necessarily display them. Another type of classification to get an overall view of the proteins identified is using PANTHER protein classification designations. These designations give an overall idea of the type of proteins that have been identified, and enrichment analysis can be performed to determine if there is an enrichment of a certain type of protein class that comes out in response to changing conditions. Not all proteins have been

assigned a panther protein classification so the interpretation of the results needs to take this into account.

1.9 Aims of the Project

Plants will adapt to stress conditions such as Pi starvation. I hypothesize that the *B. rapa* proteome will change in response to Pi starvation in such a way as to be better adapted to tolerate low Pi conditions and improve their Pi efficiency. My aim is to identify what proteins change in abundance as a part of a PSR to improve plants Pi efficiency. To determine this I will analyse how the proteome and phosphoproteome of the model organism *Arabidopsis* as well as the crop species *B. rapa* change in response to Pi starvation. The objectives of this thesis are as follows:

- Identify how the *Arabidopsis* exudate proteome and phosphoproteome changes in response to Pi starvation (Chapter 3).
- Identify how the *B. rapa* exudate proteome changes in response to Pi starvation (Chapter 4).
- Identify how *B. rapa* root proteome and root phosphoproteome changes in response to Pi starvation (Chapter 5).
- Identify how *B. rapa* shoot transcriptome, proteome and phosphoproteome changes in response to Pi starvation (Chapter 6).

Chapter 2

Methods and material

2.1 Plant Growth Conditions

2.1.1 *B. rapa* hydroponic growth conditions

Brassica rapa R500 and IMB211 seedlings were surface sterilised in 70% ethanol for 1 minute before being washed in 50% NaClO for 1 minute, followed by 4 washes in sterile water. Seeds were plated on filter paper with sterile water, left in the dark at 4 °C for 24 hours before being placed in a Panasonic MLR-352 PE Climate Chamber, set at 22°C day and 20°C night, 16 hour light, 8 hour dark cycle.

At 7 days old, seedlings were transferred to horticultural silver sand and water for another week to allow the root architecture to develop. After which they were transferred into the hydroponic growth box's, supported by sponge stoppers in Pi sufficient plus Pi (PP) media. The PP media contained (0.1 mM KH_2PO_4, 0.5 mM KOH, 0.75 mM $MgSO_4.7H_2O$, 0.03 mM $CaCl_2.2H_2O$, 0.1 mM FeNa-EDTA, 2 mM $Ca(NO_3)2.4H_2O$, 2 mM NH_4NO_3, 30 µmol H_3BO_3, 10 µmol $MnSO_4.4H_2O$, 1 µmol $ZnSO_4.7H_2O$, 3 µmol $CuSO_4.5H_2O$, 0.5 µmol $NaMoO_4.2H_2O$). When the seedlings were 3 weeks old half of the plants were transferred to Pi deficient minus Pi (MP) media, and the other half were replenished with fresh PP media. MP media omits KH_2PO_4 and replaces it with 0.13 mM K_2SO_4. The media was refreshed after a week. When the seedlings were 30 days old they were separated and placed into 50 ml falcon tubes with their roots submerged in 25 ml of media for 24 hours. After the 24 hours the media was frozen to collect the exudates, and the roots and shoots of the plants were harvested and weighed before being frozen in liquid nitrogen.

2.1.2 *Arabidopsis* swirling cultures and exudate collection

Arabidopsis Col-0 seeds were surface sterilised and 0.01 g of seeds were grown in 50 ml liquid cultures PP media which was derived from 1/2 Murashige and Skoog medium with 1 % sucrose [Wang and Liu, 2018]. Cultures were grown while gently swirling in a Panasonic MLR-352 PE Climate Chamber, set at 22°C day and 20°C night, 16 hour light cycle, light setting 4. After 5 days samples were transferred into MP media where the 1.25 mM KH_2PO_4 was replaced with 1.25 mM K_2SO_4 or were replenished with fresh PP media. After another 5 days the seedlings were harvested and frozen in liquid nitrogen ready for protein extraction, and the media was collected.

The media was frozen and freeze dried until dry, the exudates were suspended in 5 ml distilled water and dialysed for 24 hours at 4°C in distilled water using dialysis tubing (Sigma-Aldrich, catalog number: D9777). The dialysed extracts were further freeze-dried until dry then suspended in exudate buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1x PIC, 1 mM PMSF).

2.1.3 *Arabidopsis* grown on murashige and skoog medium plates

Col-0 seedlings were surface sterilised and plated on PP or MP 1/2 Murashige and Skoog medium which included 1 % sucrose, and 1 % phytoagar (Duchefa Biochemie). After leaving in the dark at 4°C for 24 hours plates were placed in a Panasonic MLR-352 PE Climate Chamber, set at 22°C day and 20°C night, 16 hour light cycle for 2 weeks before phenotype analysis. Seedlings were imaged and root lengths measured using ImageJ.

2.2 Protein extraction

2.2.1 Membrane associated proteome isolation

Plant material harvested was flash frozen in liquid nitrogen then lypholised until dry. Root material was ground to a fine powder before adding 1:4 weight to volume ratio of extraction buffer (50 mM Tris HCl pH 7.8, 500 mM Sucrose, 10% Glycerol, 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 5 mM β -glycerphosphate, 0.6% PVP, 10 mM Ascorbic acid, 5 mM DTT, 5 mM PMSF, 1x PIC), the homogenate was then filtered through MiraCloth (Merck). To separate the Membrane associated proteins the extract was centrifuged at 26,000 xg for 25 minutes, 4°C in a Optima MAZ-XP bench top ultra centrifuge, with a TLA100.3 rotor. Supernatant was spun again at

84,000 xg for 25 minutes, 4°C. Supernatant was saved as the soluble protein fraction while the pellet contains the membrane associated fraction. Pellet was resolubilised in membrane buffer (100 mM Tris HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 10% Glycerol, 20 mM NaF, 1 mM PMSF, 1x PIC). To just collect the total protein extract, the homogenate was spun at 20,000 xg for 30 minutes and the supernatant contained total protein extract.

Protein concentration was determined using a bradford assay [Bradford, 1976] with BSA used to create a standard curve to determine the protein concentrations against.

2.2.2 Protein extraction for phosphopeptide enrichment

Proteins extracted in extraction Buffer 1:4 plant weight to volume ratio (150 mM Tris HCl pH 7.5, 150 mM NaCl, 10% Glycerol, 5 mM EDTA, 10 mM NaF, 10 mM NaMo, 1% calyculin A (Thermo Scientific), 2 mM Na_3VO_4, 5 mM DTT, 1 mM PMSF, 1x PIC, 0.1% NP-40). Samples centrifuged 18,000 xg for 30 minutes, supernatant was centrifuged a second time 18,000 xg 30 minutes 4°C. Protein concentration was determined using a bradford assay before running a SDS-PAGE gel to visualise protein extract before protein digestion and phosphopeptide enrichment. 1 mg of protein extract used for phosphopeptide enrichment.

2.3 Mass spectrometry

Due to the high number of samples and MS experiments performed during this study Table 2.1 summaries the different MS methods used for the different experiments and samples, looking at the preparations, digestion type and the MS run time.

Table 2.1: Summary of experimental methods: An overview of the different experiments discussed in each chapter, summarizing the samples growth conditions, preparation and digest used before the MS run.

Experiment	Growth conditions	Preperation	Digest	MS run
Chapter 1				
Arabidopsis root exudate proteome	10 day old Col-0 grown in swirlling cultures. 5 days Pi starved compared to control. Media collected from 3 replicates.	Media collected, freeze dried and dialysed before sepperating 20 μg on 12% SDS-PAGE gel.	In-gel tryptic digest	1 hour run per sample
Arabidopsis seedlings phosphoproteome	10 day old Col-0 grown in swirlling cultures. 5 days Pi starved compared to control. 3 replicates harvested.	Seedlings collected, frozen, total proteins extracted.	1 mg of proteins FASP digested then TiO_2 phosphoenrichment	1 hour run per sample
Chapter 2				
R500 root exudate proteome	31 days old hydroponically grown, 11 days starved of Pi or maintained in sufficient Pi media. Exudates collected over 24 hours	Media freeze dryed, and proteins collected pooled together from 6 plants in 4 replicates. Proteins run on a SDS-PAGE gel	In-Gel tryptic digest	1 hour run per sample
IMB211 root exudate proteome	31 days old hydroponically grown, 11 days starved of Pi or maintained in sufficient Pi media. Exudates collected over 24 hours	Media freeze dryed, and proteins collected pooled together from 6 plants in 4 replicates. Proteins run on a SDS-PAGE gel	In-Gel tryptic digest	1 hour run per sample
Chapter 3				
R500 root membrane associated root proteome	31 days old hydroponically grown, 11 days starved of Pi or maintained in sufficient Pi media.	Root samples lypholised untill dry. Total proteins extracted then membrane proteins isolated. 20 μg of proteins run on a 12 % SDS-PAGE gel.	In-Gel tryptic Digest. Each lane fractionated into 5 pieces	1 hour run per fraction
R500 root soluble proteome	31 days old hydroponically grown, 11 days starved of Pi or maintained in sufficient Pi media.	Root samples lypholised untill dry. Total proteins extracted then membrane proteins seperated. Soluble proteins saved for digest.	500 μg FASP digest. Stage tipped.	4 hour run per sample
R500 root phosphoproteome	31 days old hydroponically grown, 11 days starved of Pi or maintained in sufficient Pi media.	Root samples lypholised untill dry. Total proteins extracted.	1 mg of proteins FASP digested then TiO_2 phosphoenrichment	1 hour run per sample

Continued on next page

Table 2.1 – *Continued from previous page*

Experiment	Growth conditions	Preperation	Digest	MS run
Chapter 4				
R500 shoot proteome	31 days old hydroponically grown, 11 days starved of Pi or maintained in sufficient Pi media.	Shoot samples lypholised untill dry. Total protein extracted.	1 mg of proteins FASP digest, ∼20 μg peptides stage tipped.	1 hour run per sample
R500 shoot phospho-proteome	31 day old hydroponically grown, 11 days starved of Pi or maintained in sufficient Pi media.	Shoot samples lypholised untill dry. Total protein extracted.	1 mg of proteins FASP digest then TiO_2 phosphoenriched	1 hour run per sample

2.3.1 In-gel protein digest

Proteins were run on 12% SDS-PAGE gel and protein bands were visualised using coomassie brilliant blue dye. The protein lanes were then fractioned into 5 pieces, each piece was cut into 2-4 mm cubed pieces of gel ready for digestion. The gel sections were de-stained in 50% ethanol and 50 mM ammonium bicarbonate (ABC) for 20 minutes at 55 °C shaking at 650 rpm, repeated three times. Gel pieces were then dehydrated in 100% ethanol for 5 minutes at 650 rpm. Protein alkylation and reduction was performed using 10 mM TCEP and 40 mM CAA for 5 minutes at 70°C. The gel peices were washed three times for 20 minutes in 50% ethanol and 50 mM ABC shaking at 650 rpm . Gel pieces dehydrated again in 100% ethanol for 5 minutes at 650 rpm. Proteins were digested by rehydrating the gels in 2.5 ng/µl trypsin with 50 mM ABC and leaving overnight at 37°C.

The following day peptides were extracted from the gels. 25% acetonitrile and 5% formic acid was added to cover the gel slices then sonicated for 10 minutes in a water bath. All excess liquid was collected and saved before repeating twice. The peptides were concentrated using a speed vac at 45 °C until volume was approximately 20 µl. The peptides were suspended in 2% acetonitrile and 0.1% formic acid to a final volume of 50 µl ready to be run on the MS.

2.3.2 FASP Digest

Protein extracts digested by Filter Aided Sample Preparation (FASP) digest to prepare the samples for MS analysis. Samples were diluted in 6 M Urea, 50 mM Tris Hcl, 75 mM NaCl. Samples added to the 0.5 ml 10 KDa cut off filter column (millepore). Samples were spun at 8,000 g for 20 minutes. Samples were washed 3

times in urea buffer centrifuged at 8,000 xg. Urea buffer was exchanged 3 times to 50 mM ABC by centrifuging at 8,000 xg. Samples were reduced and alkylated using 10 mM TCEP and 40 mM CAA in 50 mM ABC, before being centrifuged at 8,000 xg for 20 minutes. Samples were washed twice with 50 mM ABC and spun at 8,000 xg for 20 minutes. Samples were digested in the filter units using trypsin 2 µg/100 µg protein. Samples were left overnight at 37 °C to digest.

Resultant peptides were collected from the filter column by centrifuging at 8,000 xg for 20 minutes, then eluting with water and centrifuging at 8,000 xg for 20 minutes. 10 µg of peptides were allocated for stage tip to clean up before MS analysis.

2.3.3 Peptide clean up

To ensure that the peptides are clean and all excess salts have been removed a C18 stage tip was performed. C18 membranes was prepared in p200 pippette tip columns, membranes were conditioned by adding 100% methanol, followed by equilibration with 100% acetonitrile, next it was equilibrated with 2% acetonitrile, 0.1% trifluoroacetic acid (TFA). 10 µg of digested peptides suspended in 2% acetonitrile with 0.1% TFA were put through the prepared C18 membrane. Peptides were washed with ethyl acetate, 1% TFA, then washed again with 2% acetonitrile, 0.1% TFA. Peptides were eluted from the C18 membrane using 80% acetonitrile. Once eluted the acetonitrile was removed by speed vac. Peptides were re-suspended in 2% acetonitrile and 0.1% TFA ready for MS.

2.3.4 Phosphopeptide enrichment

After 1 mg of proteins underwent a FASP tryptic digest they were dried in a speed vac untill dry. Peptide samples were re-suspended in 1 mL of Resuspension Buffer (5 mM HEPES, 80% ACN, 5% TFA), vortexed and sonicated for 5 minutes. Titanospere titanium dioxide TiO_2 5 µm beads 5020-75000 (GL Sciences Inc. Japan), enough for 2 enrichments of 1:2 mg of peptide to mg of beads ratio, diluted to 1 mg of beads in 10 μl of resuspension buffer plus 20 mg/mL solution of 2,5-dihydroxybenzoic acid (DHB), and mixed for 10 minutes at 600 rpm. Prepared beads added to peptide samples and mixed end over end for 1 hour. Samples centrifuged at 2000 xg for 2 minutes, supernatant removed for a second enrichment. A C8 membrane was packed into pipette tips to make C8 column. Column was prepared for samples by conditioning using 100% ethanol, followed by equilibrating with organic solvent; 100% acetonitrile, then equilibrated with aqueous solution; 2% acetonitrile and 0.1% TFA. Beads from the 2 enrichment steps were suspended in 50 µL Wash buffer 1

(10% Acetonitrile, 5% TFA) and transferred to C8 stage tip, centrifuged at 2000 rpm for 2 minutes. Washed with 100 µL Wash buffer 2 (40% Acetonitrile, 5% TFA) centrifuge at 2000 rpm for 2 minutes. Washed again with 100 µL Wash buffer 3 (60% Acetonitrile, 5% TFA), centrifuge at 2000 rpm for 2 minutes. The phosphopeptides were eluted off the beads and C8 column with 20 µL Elution buffer 1 (5% ammonium hydroxide) then 20 µL Elution buffer 2 (12 % ammonium hydroxide, 25% Acetonitrile) centrifuging 2000 xg for 4 minutes. Combine elutions then speed vac to less than 5 µL to remove acetonitrile, peptides were re-suspended in 2% acetonitrile and 0.1% TFA ready to be run on the MS.

2.3.5 Protein detection

Samples were separated with UltiMate 3000 series high performance liquid chromatography (HPLC) using Nano Series standard reverse phase 50 cm columns, the columns are packed with C18 coated silica particles, allowing peptides to be sepperated based on their hydrophobicity by eluting with an increase acetonitrile concentartion gradient from 8% to 25% acetonitrile over time, the LC separation time is different based on the experiment type stated in Table 2.1. Eluting peptides were analysed with nano liquid chromatography electrospray ionisation-tandem mass spectrometry with the Ultimate 3000 Orbitrap Fusion (Thermo Scientific). The mass spectrometer was operated in positive ion mode, and in data dependent mode. Peptide precursors survay scans were performed in the orbitrap and MSMS was performed by isolation using the quadrapole and with higher-energy collision dissociation (HCD) fragmentation, and rapid scan MS analysis performed in the ion trap.

2.3.6 Data analysis

MaxQuant peptide assignment and label free quantification

Raw files generated from the MS were used for searches to be matched to *Arabidopsis* Col-0 proteome from Uniprot, or *B. rapa* yellow sarson Z1 proteome from genoscope (http://www.genoscope.cns.fr/genomes)[Boutte et al., 2020, Istace et al., 2021] using MaxQuant (MQ) version 1.6.2.6 [Cox and Mann, 2008]. The Z1 proteome was annotated by Richard Stark and Laura Baxter from the University of Warwick bioinformatics research technology platform, mapping the Z1 identifiers to UniProtKB unique identifiers, then running DIAMOND sequence alligner to get the best *Arabidopsis* match of each identifier. MQ Searches were performed with trypsin as the digestion enzyme with up to 2 missed cleavages, and a parent ion

mass tolerance of 4.5 ppm. Match between runs was used during MQ searches. Variable modification assigned to oxidation of methionine, phosphorylation on S, T and Y, and acytylation. Fixed modification set as carbamidomethylation of cystine. Label free quantification (LFQ) searches were used for quantitative analysis, and identification FDR set to 0.01.

Perseus analysis of lable free quantification

MQ generated protein group.txt files with LFQ intensity values were inputted into Perseus version 1.6.10 for analysis. Reverse searches, potential contaminants and only identified by site hits were filtered from the data. Samples were grouped by growth condition, LFQ intensity values $\log_2$ transformed, If there were not more that at least 3 valid values in one group then that hit would be removed from the data allowing for the filtering of low confidance anomalous proteins from within the data sets. Any missing values were then imputed from the normal distribution of observed values. Intensities were normalised using the sum intensity values of the proteins. When protein groups were identified where it wasnt possible to determine the specific protein identified from unique peptide hits, then the top hit protein in the majority protein ID catagory was used to identify the protein within the group. PCA, volcano, and cluster heat maps could then be generated and lists of differentially abundant proteins were determined for further analysis and discussion [Tyanova et al., 2016]. Student sample t-test was used to determine proteins significantly changed in abundance from the $\log_2$ LFQ intensities, with an FDR value set at 0.05, and an s0 value set at 0.1. s0 is a minimal fold change, and FDR is the negative log of the student t-test p-value. There is no general norm for determining proteins significantly different in abundance according to the Molecular and Cellular Proteomic guidelines.

Perseus analysis of Phosphopeptides

MQ generated Phospho(STY)Sites.txt file was loaded into Perseus, and intensity 1, 2 and 3 for each sample was imputted. Reverse searches and potential contaminants were filtered from the data, and any phosphosite localisation scores less than 0.75 were also filtered. Samples were grouped by growth condition, LFQ intensity values $\log_2$ transformed, If there were not more that at least 3 valid values in one group then that hit would be filtered from the data. Any missing values were imputed from the normal distribution. PCA plots, volcano plots and heat maps could then be generated, and differentially abundant phosphopeptides determined.

2.4 RNA-Seq

2.4.1 RNA Extraction

The shoot RNA of 31 day old R500 plants Pi starved for 11 days verses control were extracted using the Spectrum Plant Total RNA kit (Sigma). 10 mg of dried plant material per sample was used for extraction. RNA concentration was checked using Nanodrop 1000. The quality of the RNA was checked using the Warwick Genomics Agilent Bioanalyser to ensure the RNA integrity number was greater than 7. 3 RNA Samples per Pi condition that passed the quality check were sent to Novogene UK for mRNA-seq analysis.

2.4.2 RNA-seq analysis

RNA sequencing using Illumina PE150 (Novogene). Poly A enrichment for mRNA library preparation, sequencing was paired end and unstranded with a read length of 150 BP, and a depth of sequencing of at least 20 million paired reads per sample. Raw data was obtained in the form of .fq.gz files for each pair of each sample.

2.4.3 Bioinformatics and data processing

FastQC was used for the initial quality check of the RNA-seq raw reads, which were then subjected to trimming and adapter removal using FastP [Chen et al., 2018], trimmed reads underwent fastQC again for quality control. Alignments of paired end reads was performed by STAR [Dobin et al., 2013], index files generated with the *B. rapa* R500 genome and annotation files from CoGe (v1.2, id52010) [Lyons and Freeling, 2008], with the sjdbOverhang parameter set to 149 (read length -1). Raw counts data for 33,030 genes in the *B. rapa* genome were obtained, and analysis for the differentially expressed genes was performed in R using DESeq2 package [Love et al., 2014], genes were considered significantly differentially expressed if they exhibited at least a $\log_2$ fold change of 1, and a Bonferroni-corrected P-value < 0.05 from a walds test.

2.5 Protein analysis

The *B. rapa* Z1 database was used to obtain protein identifiers from the MS data analysis for the *B. rapa* samples, these identifiers used Uniprot to obtain protein information [The UniProt Consortium, 2021]. Uncharacterised proteins protein information was determined using the proteins *Arabidopsis thaliana* TAIR

10.1 homologues [Kaul et al., 2000], and the *B. rapa* Chiifu V1.5 homologies were determined using Brassicaceae Database BRAD V3.0 (http://brassicadb.cn) and BLAST (sequenceServer 1.0.14)[Priyam et al., 2019, Wang et al., 2011b]. The Chiifu homologues were used to generate protein identifiers to be used for enrichment analysis. The presence of signal peptides and predicted subcellular locations of proteins was predicted using TargetP 2.0 (https:// services.healthtech.dtu.dk/ service.php?TargetP-2.0) [Armenteros et al., 2019]. Transmembrane domains were predicted using TMHMM - 2.0 (https://services.healthtech .dtu.dk/ service.php? TMHMM-2.0) [Möller et al., 2001].

Phosphomodification motif analysis was performed using MoMo on Meme suite. Motif-x algorithm was used on 13 residue size sequence window of the detected phosphorylation sites, background peptides taken from the *Arabidopsis* context sequence for Col-0 phosphoproteome motif analysis, and background peptides taken from *B. rapa* context sequence for *B. rapa* R500 motif analysis. P-value threshold set at $< 1e^{-}6$ [Cheng et al., 2019].

2.5.1 Enrichment analysis

Proteins that were determined significantly differentially abundant and genes considered significantly differentially expressed underwent enrichment analysis using PANTHER16.0, to query over or under representation of GO terms as well as PANTHER protein classification. *B. rapa subspecies pekinensis* was used as a background reference for the *B. rapa* data and *Arabidopsis thaliana* was used as a background reference for the *Arabidopsis* data (Chapter 3). A Fisher exact test with Benjamini-Hochberg FDR correction <0.05 was used to determine significantly enriched terms [Mi et al., 2019]. When multiple terms were enriched as a part of the same hierarchy branch, one overall term was chosen to represent them.

2.5.2 Phylogenetic analysis of protein groups

Protein sequences taken from uniprot aligned using Jalview2.11.1.4 clustal omega, set with jalview default settings [Sievers et al., 2011]. Alignments put into in MEGA5.0 to create phylogenetic tree [Kumar et al., 2018, Stecher et al., 2020] using neighbor joining tree method [Saitou and Nei, 1987] set at 500 bootstraps, with the branch distance calculated with the p-distance method [Nei and Kumar, 2000], uniform rates among sites set, and pairwise deletion of gaps missing data treatment.

2.6 Malachite green colorimetric assay

50 mg dry plant material homogenized in 3 ml water and 50 µL 5 M H_2SO_4, samples centrifuged at 10000 xg for 10 minutes. The supernatant was used to determine the levels of free Pi within samples using a malachite green phosphate assay kit (Sigma), measuring the absorbance at 620 nm.

2.7 Phosphatase detection activity assay

2.7.1 In-gel phosphatase assay

The in gel acid phosphatase activity assays were performed from Wang and Liu [2018] method. 2.5 µg of proteins were separated on a 10 % native PAGE gel. The gels were run at 4 °C for 3 hours at 90 volts. Once run the gels were washed in deionized water for 10 minutes. After that they were equilibrated in reaction buffer (50 mM NaAc, 10 mM MgCl2, pH 4.9), for 15 minutes 5 times at 4 °C. Gels were then incubated for 3 hours at 37 °C in APase profiling buffer (reaction buffer, 0.3 mg/ml β-naphthyl acid phosphate (sigma), 0.5 mg/ml Fast black K (sigma)). After the incubation the gels were washed for 10 minutes in deionized water and then were imaged, red pigmentation indicates APase activity.

2.7.2 5-Bromo-4-chloro-3-indolyl phosphate root surface phosphatase assay

Root surface staining for APase activity using 5-Bromo-4-chloro-3-indolyl phosphate (BCIP)(Sigma-Aldrich). After plants had been grown of PP or MP plates, BCIP overlay solution was added (50 mM NaAc, 10 mM $MgCl_2$, 0.5 % agar, 0.1 mg/ml BCIP) to the roots, plants were incubated for 2 hours as 22 °C before being imaged, blue coloration indicates phosphatase activity.

2.7.3 Root associated APase assay

Arabidopsis seedlings grown on 1/2 Murashige and Skoog medium in either PP or MP conditions for 10 days. Roots were measured then severed form the plant, 2 roots were submerged in 700 µL APase reaction buffer (10 mM $MgCl_2$, 50 mM NaAc, 70 ug/ml Para-nitrophenyl phosphate (pNPP) (Sigma-Aldrich)) for 1-4 hours at 37 °C. 100 µL of 0.4 M NaOH was added to halt the reaction, then the solutions absorbance at 410 nm (A410) was measured using a spectrophotometer and used to calculate the root-associated APase activity as A410/cm root/h.

Chapter 3

Quantitative Proteomics of Phosphate Starved *Arabidopsis thaliana*

3.1 Introduction

3.1.1 *Arabidopsis* and phosphate stress

The lack of any essential nutrient has a profound effect on the growth and development of plants, P in one such essential macronutrient which can limit a plants growth when accessible Pi is unavailable. When in Pi limiting conditions *Arabidopsis* growth will be effected and the plant will induce a phosphate starvation response (PSR) in an effort to improve the availability and acquisition of available Pi sources. These responses are varied and work to improve *Arabidopsis* Pi efficiency during low Pi conditions, either by modifying the uptake of Pi or the utilisation of available Pi. *Arabidopsis* is known to alter root architecture; with decreasing primary root length and increased lateral roots to improve top soil foraging of Pi [Lynch and Brown, 2001]. An increase in Pi transporters on the plasma membrane to maximise uptake of available Pi [Puga et al., 2017], and the redistribution of stored Pi within the plants improves Pi efficiency [Chang et al., 2019, Mlodzinska and Zboinska, 2016, Poirier et al., 1991]. Proteomic changes also occur within the plants as a PSR to improve Pi efficiency, displaying an increased abundance of protein, nucleic acid and lipid metabolism proteins and oxidative stress proteins [Chevalier and Rossignol, 2011].

Research on *Arabidopsis* has provided insight into a plants responses to Pi starvation, suggesting how some proteins that are responsive to Pi stress can be utilised to further improve the plants response to low Pi conditions, such as overexpression of Pi responsive AtPAP10 protein [Wang et al., 2011a] that led to increased growth during low Pi conditions. The overexpression of different PHT1 transporters in *Arabidopsis* has also yielded some improvements to phosphate uptake in both high and low Pi conditions [Gu et al., 2016]. Therefore identifying Pi responsive proteins provides some promising targets for improving plant growth during phosphate stress.

3.1.2 Root exudates of phosphate starved *Arabidopsis*

Roots will secrete various compounds into their surrounding environment to better adapt to the local conditions, such as improving the accessibility of nutrients like Pi and responding to stress. The secretion of proteins such as acid phosphatases and RNase [Abel et al., 2000, Raghothama and Karthikeyan, 2005] improve phosphate mobility and increase availability for the plants [Wang and Liu, 2018], and the excretion of other compounds such as organic acids help to mold the environment to better suit the plants needs. I am specifically interested in the protein exudates, and how they change depending on whether *Arabidopsis* is grown Pi sufficient verses Pi deficient conditions to have a better insight into what proteins could improve the Pi availability as a part of a PSR.

The secretion of proteins in response to Pi stress has been researched before, but the use of 2D electrophoresis and MALDI-TOF MS limited the identification and analysis of secreted proteins [Tran and Plaxton, 2008]. A 2D-gel-MALDI MS approach doesnt provide as in depth coverage of proteins present within a sample compared to a LC-MS approach. Therefore, I have been able to undertake an in depth coverage of the excreated proteome. Other studies on the topic of secreted proteins in response to phosphate stress have specifically found individual proteins or protein family's secreted rather than an overall proteomic change. For example, the study of the PAP family proteins that are secreted, AtPAP10, 12, 25, and 16 have been classified as secreted phosphate starvation induced PAPs, a vegetative storage protein (AtVSP3) is also characterised as secreted and acts as an acid phosphatase responsive to phosphate starvation [Sun et al., 2018, Tran et al., 2010, Wang and Liu, 2018].

The effects of phosphate stress on *Arabidopsis* plants and their root secretions is not a new topic, but in this chapter I present an in depth coverage of the excreated

proteome, and present new data on proteins secreted by *Arabidopsis* seedlings during Pi stress and offer an in depth insight to the topic. This work also plays a role in establishing a system for the study of *Brassica* response to Pi stress. The use of *Arabidopsis* as a model for Pi uptake in *Brassica* works well as they both do not form mycorrhizal associations to interfere with the plants responses to Pi stress conditions, but they are still different species therefore are not expected to respond in exactly the same way.

3.1.3 The role of protein phosphorylation in phosphate starved *Arabidopsis*

Protein phosphorylation is a reversible PTM, it is the addition of a phosphoryl group to an amino acid residue. Kinase enzymes catalyse the phosphorylation while phosphatases will cause the dephosphorylation of proteins. The activity of a protein is often dependent on it "being switched on or off" via phosphorylation [Ardito et al., 2017]. *Arabidopsis* encodes more than 1000 different kinase proteins and around 200 phosphatase proteins, therefore there is the potential for many phosphorylation events to occur [Ajadi et al., 2020, Kersten et al., 2006].

Some kinase enzyme activity is also dependent on its phosphorylation status, therefore, protein kinases involved in signalling events such as in response to Pi starvation could be identified by phosphoproteomic analysis, and potentially indicate what kinases are active during Pi stress which are not during control conditions. Phosphoproteomics is a useful tool that can be employed in an attempt to identify phosphorylated proteins, which could then be linked to signalling events during stress conditions.

Despite quite extensive coverage of phosphoproteomics in response to other environmental conditions in *Arabidopsis*, especially immune response pathways as well as nitrogen deficiency, the study of phosphoproteomics in response to Pi deprivation has been sorely neglected. There have been some attempts, the use of *Arabidopsis* suspensions cells to observe changes in the phosphoproteome in response to Pi availability by Mehta et al. [2020] for one, but to my current knowledge whole seedlings phosphoproteomics in response to Pi starvation has not been published previously. There has been some work done on specific targets of phosphorylation events in response to Pi starvation. Phosphate transporters undergo phosphorylation, acting to modulate the transporters exit from the ER and localisation to plasma membranes, resulting in a decreased abundance of Pi transporters localized to plasma membranes during a PSR [Bayle et al., 2011]. To get a broader coverage

of changes in *Arabidopsis* phosphoproteome in response to Pi starvation, as a part of this chapter I undertook phosphoproteome analysis of *Arabidopsis* seedlings during Pi starvation.

3.1.4 Aims

The aim of this chapter was to identify *Arabidopsis* exudate proteins and signalling proteins, and determine how they change in response to Pi starvation. To achieve this aim *Arabidopsis* Col-0 seedlings were grown in either Pi sufficient or Pi deficient conditions to induce a phosphate starvation response. These samples were then used for the following analysis:

- Measure the physiological response of *Arabidopsis* during Pi starvation compared to control conditions; root length, root phosphatase activity.
- Quantitative analysis of *Arabidopsis* root exudate proteome in response to Pi starvation.
- Quantitative analysis of *Arabidopsis* phosphoproteome in response to Pi starvation.

3.2 Results

In order to analyse the proteomic changes in *Arabidopsis* plants in response to Pi starvation I needed to establish conditions to trigger a Phosphate Starvation Response (PSR). Col-0 were grown on Pi sufficient or Pi deficient plates to measure the root responses to check that a PSR was being initiated, as changes in root length correlates with *Arabidopsis* response to Pi starvation. To analyse proteomic changes, Col-0 seedlings were grown in Pi sufficient liquid media for five days before being either moved to Pi deficient media or replenished with Pi sufficient media for a further five days. The media was harvested for exudate collection and exudate proteomic analysis. The seedling proteins were extracted for phosphopeptide enrichment for phosphoproteomic MS analysis (Fig.3.1).

3.2.1 Phosphate starvation induced response in *Arabidopsis* leads to physiological changes and increased APase activity

Plants will induce a variety of physiological responses to adapt to low Pi conditions, such as changes in primary root length (PRL), plant fresh weight (FW), and APase activity which has been shown in previous studies to increase in response to low

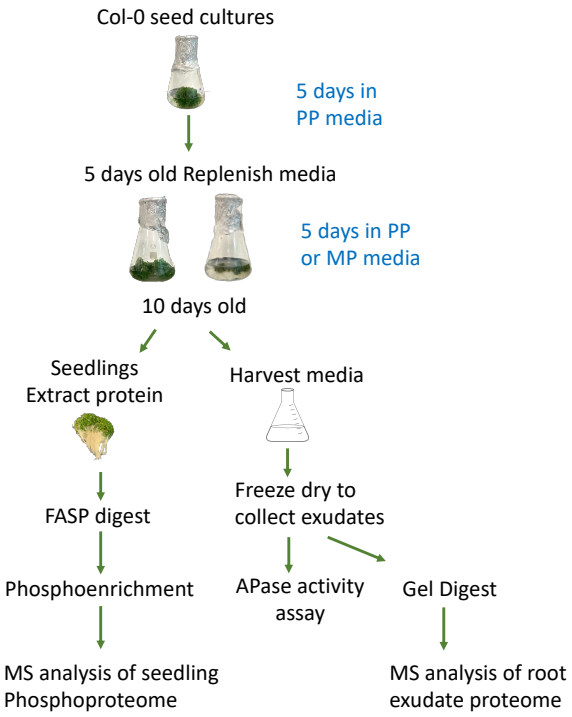

Figure 3.1: Preparation of *Arabidopsis* exudates and phosphoproteome: Col-0 *Arabidopsis* plants grown in plus Pi (PP) swirling cultures for 5 days. Three replicates were moved to minus Pi (MP) media while another three replicates were replenished in PP media and grown for another five days. The seedlings were harvested and the total proteins extracted to be used in a FASP digest then phosphopeptide enrichment for MS analysis of the phosphoproteome. The media the plants were grown in was collected, freeze dried, and the exudate proteins used for activity assays or MS analysis.

Pi conditions [Wang and Liu, 2018]. In order to determine that a PSR has been induced in Col-0 *Arabidopsis* seedlings grown in Pi deficient conditions compared to Pi sufficient conditions, a variety of responses were measured.

Ten day old Col-0 seedlings grown on Pi deficient or sufficient plates were imaged and the PRL measured, they showed that the Pi starved seedlings had significantly shorter PRL than those of the control seedlings by an average of 1.2 cm (Fig. 3.2A). The root surface APase activity of the seedlings grown on Pi deficient compared to Pi sufficient plates was also measured. A root surface BCIP assay was performed and the seedlings imaged, a greater blue coloration of roots gave a qualitative indication that there was higher APase activity on the roots of Pi starved seedlings, these seedlings imaged also showed less shoot material and darker green pigmentation of the leaf material (Fig. 3.2B). The root surface APase activity was also quantified using a pNPP colorimetric assay, when acted upon by an acid phosphatase pNPP produces a yellow pigmentation that can be measured at 410 nm, this showed signif-

icantly higher APase activity on the root surface of seedlings grown in Pi deficient conditions compared to the control growth conditions (Fig. 3.2B). A PSR was induced in the ten day old seedlings grown on Pi deficient plates indicated be an increase in root surface APase activity, and a decrease in PRL.

Swirling cultures were also used to determine if a PSR was induced in ten day old Col-0 seedlings after five days of Pi starvation. Approximately 0.01 g of seeds were grown in Pi sufficient liquid media for 5 days before being starved of Pi for a further five days, or were maintained in control conditions. The resultant seedlings were harvested and the FW measured. The Pi starved seedlings were significantly lighter and visually smaller than the seedlings grown in control conditions (Fig. 3.2C) indicating a PSR was induced and that Pi starvation hinders Col-0 growth.

The proteins were extracted from the seedlings grown in the swirling cultures and the exudate proteins were collected from the growth media. These proteins were then used to test for APase activity using an in-gel APase activity assay, where the gel was incubated in an activity buffer containing BNAP and Fast Black K, so phosphatase activity results in a red coloration. The gels imaged for the seedlings protein extracts (Fig. 3.2D) showed increased coloration of the Pi starved samples at approximately molecular weight 180 KDa and 100 KDa. The root exudates APase activity (Fig. 3.2E) showed darker coloration in the Pi starved samples than in the control samples at approximately molecular weight 80 KDa and 100 KDa. Therefore these results confirm that there was greater APase activity in the Pi starved seedling proteins and exudates than in the control plants, and at different molecular weights indicating different APase proteins in the seedlings and in the exudates were responsible for the increased activity.

The data presented in Fig.3.2 confirms that a PSR was induced in ten day old Col-0 seedlings after five days in Pi deficient media. A PSR leads to a significant change in the physiology and APase activity of *Arabidopsis* seedlings, with a decrease in PRL and FW, and an increase in APase activity on the root surface, root exudates and protein extracts. The root exudates were prepared for protein identification using MS, and the seedling proteins for quantitative phosphoproteomics of Pi starved *Arabidopsis*.

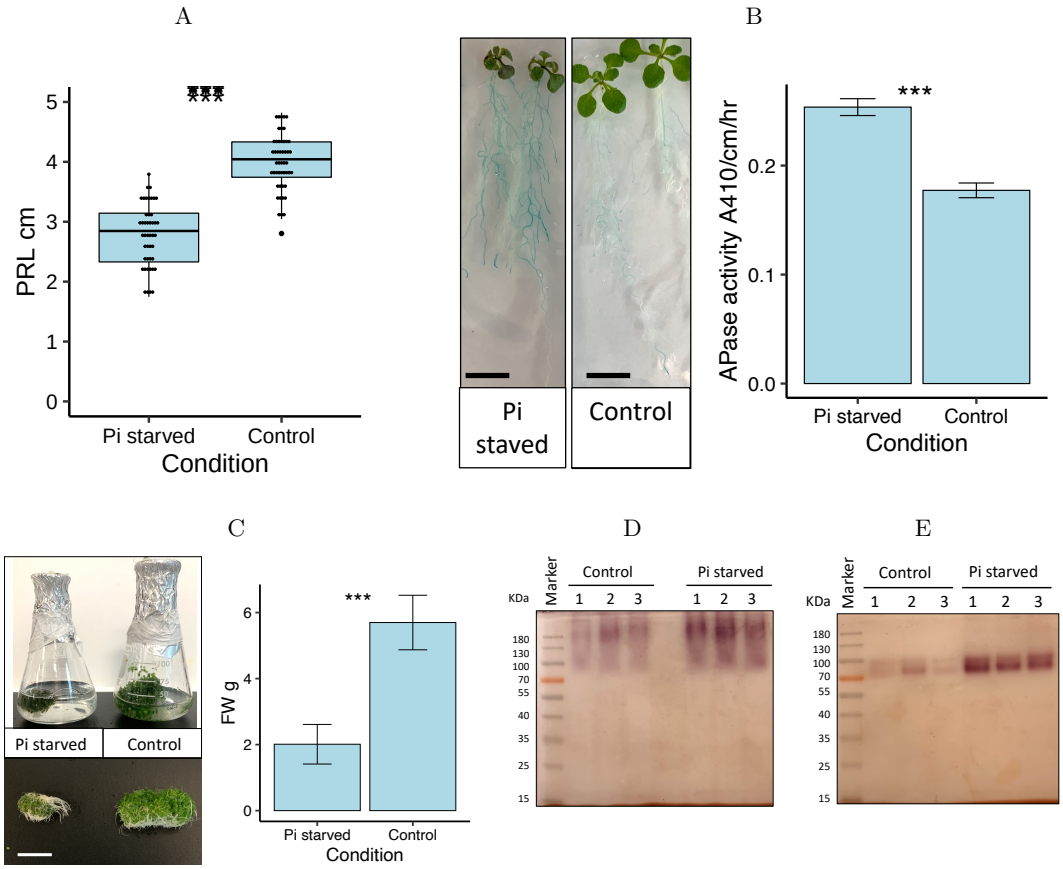

Figure 3.2: Summary of PSR phenotypes in *Arabidopsis*: 10 day old Col-0 seedlings Pi starved compared to control. A) PRL of the seedlings grown on either PP or MP plates, n > 40. B) Root surface APase activity of seedings grown on either PP or MP plates. Images from the BCIP assay where blue coloration indicates APase activity, and graph from colormetric pNPP root surface APase assay quantified, n > 8. C) Image of representative swirling cultures from the growth of approximately 0.01 g Col-0 seeds after 5 days of Pi starvation and the fresh weight of the seedlings measured, n > 6. D, E) In-gel APase activity assay on the (D) whole seedlings protein extracts and (E) exudates proteome of Col-0 swirling cultures, 2.5 ug of proteins run per sample and incubated in APase activity buffer, red bands depict APase activity. Standard 2 sample t-test used to measure significant differences in values: ***; $p < 0.001$. Scale bars represent 1 cm in length.

3.2.2 PSR in *Arabidopsis* leads to a significant change in the exudate proteome with an increased abundance of APase, and carbohydrate and lipid metabolic proteins

The proteins secreted from *Arabidopsis* roots will change in response to low Pi conditions. The changes in the root exudates are expected to enable the plant to adapt to the low Pi conditions to improve the plants Pi uptake and utilisation efficiency. The secreted proteins were collected from *Arabidopsis* Col-0 seedlings that were grown in swirling cultures for 10 days, 3 replicates were Pi starved for 5 days to induce a PSR, while 3 replicates were maintained in control conditions. The

media was then harvested and the exudates collected for the secreted proteins to be identified by MS for quantitative analysis.

After the MS analysis a total of 23,968 MS spectra were recorded, which were assigned to 1,144 proteins. The proteins were then filtered by the removal of low confidence anomalous proteins, by filtering potential contaminants, reverse hits, and filtered so that the protein had to be identified in all three replicates of at least one Pi condition to be considered confidently present, this left 499 proteins identified in the exudate proteome for further analysis.

Using the LFQ intensity values the identified proteins were further analysed to determine any changes in the exudates proteome in response to Pi starvation. A principal component analysis (PCA) was performed (Fig. 3.3A) and shows a distinct separation of the Pi starved samples to the control samples, with a component 1 value of 56%, indicating a difference in the exudate proteomes in response to Pi starvation.

A standard two sample t-test was performed on the LFQ intensities to determine significantly differentially abundant proteins in response to Pi starvation, using an FDR value of 0.05 and an s0 value of 0.1. A volcano plot was made to represent this data (Fig. 3.3B), points in red show the significantly differentially abundant proteins in a $\log_2$ fold change difference with Pi starved sample intensities minus the control sample intensities, plotted against the -log p-value. Of the 499 proteins identified in the exudates 142 were significantly differentially abundant, 71 were more abundant while 71 were less abundant in Pi starved root exudates, this shown that 28.5% of the proteins identified in the exudate proteome changed significantly in response to Pi starvation. The proteins determined to be differentially abundant were selected and the $\log_2$ LFQ intensities normalised using z-score then used to create a heat map (Fig. 3.3C), the samples clustered clearly based on the Pi conditions with a clear distinction between the proteins more and less abundant in the Pi starved exudates. These results show that there is a significant difference in the exudate proteome of Col-0 plants during Pi starvation compared to Col-0 plants grown in sufficient Pi conditions.

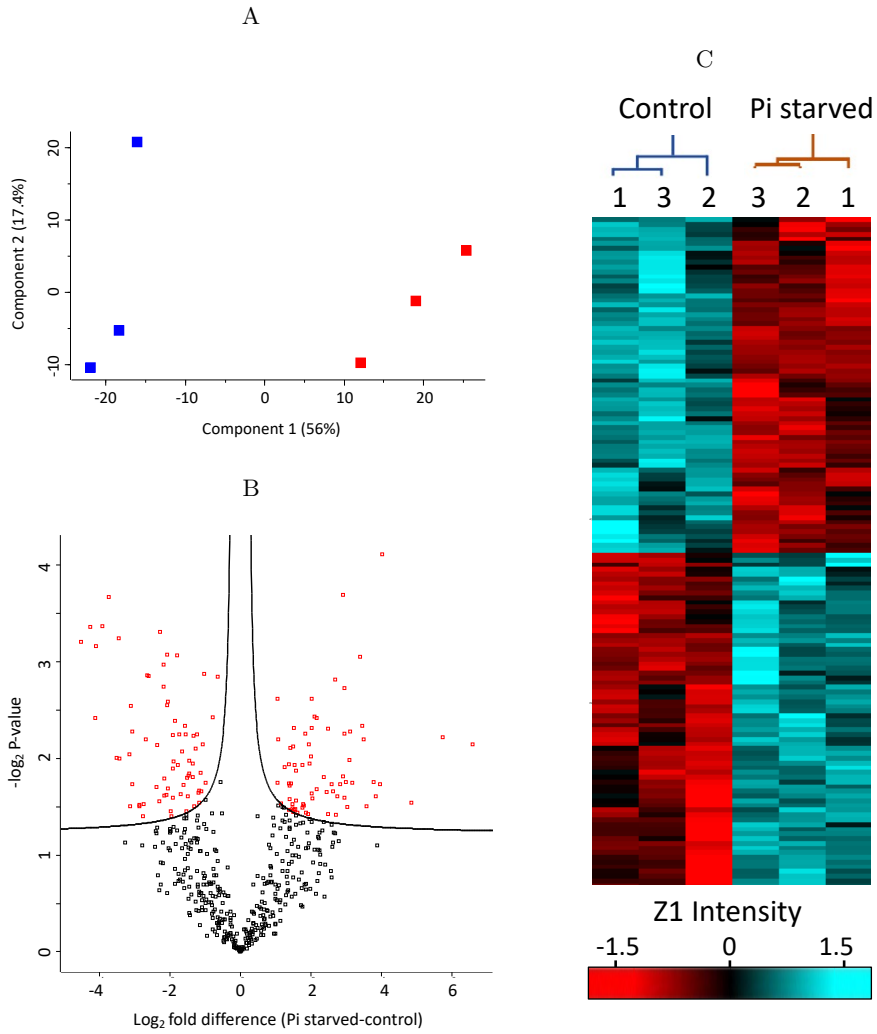

Figure 3.3: Quantitative analysis of *Arabidopsis* exudate proteome in response to Pi starvation: The proteins identified in the exudate samples were analysed in Perseus. A) PCA plot of exudate proteins, samples from Pi starved exudates highlighted in red, and control samples are highlighted in blue. B) Volcano plot of exudate proteins, using a student two sample t-test to identify proteins significantly changed in abundance between conditions, $\log_2$ fold change is plotted against -log P-value. Red points show proteins (142) which are significantly changed in abundance in response to Pi starvation. FDR: 0.05 s0: 0.1. C) Heat map of the 142 significantly changed proteins during Pi starvation. Euclidean clustering of the LFQ $\log_2$ intensity values normalised using Z-score for graphical representation. There are three replicates in each condition.

The differentially abundant proteins identified within the exudate proteomes in response to Pi starvation were used for further analysis to see what types of proteins change. The presence of a signal peptide (SP) is a good indicator that the protein is really secreted, rather than being identified from sloughed off protein material from damage caused during growth. To determine SP presence and possible localisation of identified proteins based on predicted chloroplast or mitochondrial transit peptide

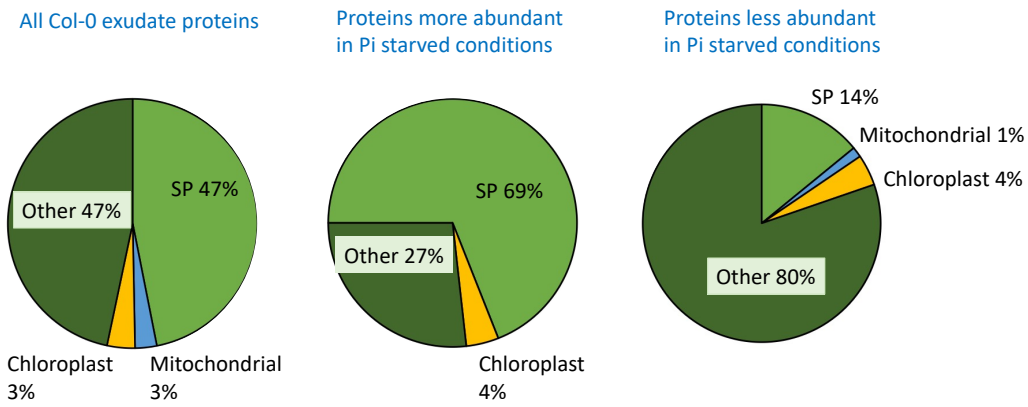

Figure 3.4: *Arabidopsis* exudate proteome predicted localisation: The proteins identified in the *Arabidopsis* exudate samples were analysed with TargetP 2.0 to predict possible protein localisation. Showing all 499 exudate proteins identified, and the 71 proteins which were determined to be significantly more abundant, and the 71 proteins significantly less abundant in response to Pi starvation.

presence, the proteins were analysed using TargetP2.0, and the relative proportions of predicted localisation are displayed in pie charts (Fig. 3.4). Of all the 499 exudate proteins identified 47% showed the presence of a SP. The proteins less abundant in Pi starved exudates showed only 14% had a SP, while the more abundant proteins in Pi starved exudates showed that 69% contained a SP, this increase in proteins identified with predicted SP could indicate an increase in secreted proteins during Pi starvation. The proteins quantified from the exudates showed that the Pi starved exudates had an average total of 19 μg/g of plant FW collected, while the control plant exudates showed an average total of 7 μg/g of plant FW collected. These results indicate that there was a greater amount of secreted proteins in Pi starved Col-0 exudates compared to control exudates, this could indicate that a PSR in *Arabidopsis* leads to an increase in the amount of secreted proteins as well as a change in the composition of the exudate proteome.

A subset of the exudate proteins identified that change in abundance in response to Pi starvation are represented in Table 3.1. The table displays the Uniprot ID, protein name, protein score, the $\log_2$ FC and Target P results. The proteins displayed are grouped under different protein groups or functions. Multiple phosphatase proteins, which includes 3 PAP proteins (PAP1, 17 and 26) increase in abundance during Pi starvation, as do various proteins involved in carbohydrate and lipid metabolic processes, as well as other proteins that could play a role in improving Pi efficiency as a part of a PSR such as ribonuclease protein, and defence proteins.

Table 3.1: *Arabidopsis* exudate proteins significantly changed in response to Pi starvation: A subset of DAP which were identified in the exudate samples are shown in this table and grouped under protein families/processes. Protein ID and protein name from uniprot, MQ protein score, The fold change (FC) of $\log_2$ LFQ intensity values Pi starved minus control, positive values indicate increase abundance within Pi starved exudates, negative values indicate decreased abundance. TargetP used to predict possible localisation and presence of SP.

Protein ID	Protein name	Score	$\log_2$ FC	-log P-value	TargetP
Carbohydrate metabolic process					
AT1G55120	Beta-fructofuranosidase	86.6	4	4.1	SP
AT4G19810	Class V chitinase	323.3	4	1.7	SP
AT4G34480	Glucan endo-1,3-beta-glucosidase 7	145.9	3	2.7	SP
AT1G51470	Myrosinase 5	25.2	2.8	1.6	SP
AT1G47600	Beta glucosidase 34	53.4	2.6	1.7	SP
AT5G14650	Pectin lyase-like superfamily protein	225.3	1.9	1.4	SP
AT3G55260	Beta-hexosaminidase 1	40.5	1.8	1.9	SP
AT4G23500	Pectin lyase-like superfamily protein	118.9	1.6	1.5	SP
AT4G26140	Beta-galactosidase	88.3	1.5	2.3	SP
AT3G13790	Glycosyl hydrolases family 32	47.6	1.4	1.5	SP
Phosphatase					
AT5G34850	Bifunctional purple acid phosphatase 26	181.5	3.1	2.3	SP
AT1G13750	Probable inactive purple acid phosphatase 1	18	2.9	1.6	SP
AT3G17790	Purple acid phosphatase 17	15	2.4	1.8	SP
AT4G29690	Alkaline-phosphatase-like	7.2	2.2	2.4	SP
AT4G29680	Alkaline-phosphatase-like	33.4	2	2.3	OTHER
Lipid metabolic process					
AT2G38530	Non-specific lipid-transfer protein 2	14.9	2.1	2.4	SP
AT4G26690	Glycerophosphodiester phosphodiesterase GDPDL3	234.9	1.4	1.7	SP
AT5G55480	Glycerophosphodiester phosphodiesterase GDPDL4	174.2	1.1	2.2	SP
AT4G36945	PLC-like phosphodiesterases superfamily protein	259.6	1.1	1.7	SP
AT1G29670	GDSL esterase	251.4	-2.1	1.6	SP
AT4G22490	Lipid-transfer protein	60.5	-2.4	1.7	SP
AT2G10940	Lipid-transfer protein	9.2	-4.1	3.2	SP
Misc					
AT5G06860	Polygalacturonase inhibitor 1	95	5.8		SP
AT4G19810	Class V chitinase	323.3	4	1.7	SP
AT2G02990	Ribonuclease 1	323.3	3.8	1.6	SP
AT1G78850	EP1-like glycoprotein 3	268.3	3.6	1.5	SP
AT1G21670	DPP6 amino-terminal domain protein	26.4	3.5	2.3	SP
cell wall modifying					
AT5G06860	Polygalacturonase inhibitor 1	95	5.8	2.2	SP
AT3G13790	Glycosyl hydrolases family 32	47.6	1.4	1.5	SP
AT4G25820	Xyloglucan endotransglucosylase	133.5	-3.5	2	SP
AT5G57530	Probable xyloglucan endotransglucosylase	28	-4.1	2.4	SP
AT5G57540	Putative xyloglucan endotransglucosylase	32.5	-4.3	3.4	SP
Defence/detoxifying					

Continued on next page

Table 3.1 – *Continued from previous page*

Protein ID	Protein name	Score	log_2 FC	-log P-value	TargetP
AT5G05340	Peroxidase 52	323.3	4.9	1.5	SP
AT1G21880	LysM domain-containing	6	3.2	1.8	SP
AT2G17120	LysM domain-containing	8.8	3	1.5	SP
AT2G37130	Peroxidase	62.3	-2.1	1.7	OTHER
AT1G30870	Peroxidase 7	78.6	-2.3	1.6	SP
AT1G07890	L-ascorbate peroxidase	37.4	-2.7	2.2	OTHER
Proteases					
AT2G24200	Leucine aminopeptidase 1	323.3	2.5	2.3	OTHER
AT3G18490	ASPARTIC PROTEASE	86.3	-3.1	1.7	SP
AT5G10770	Aspartyl protease family protein	49.8	-2.8	1.4	SP

To determine if there is a change in the overall functions and processes of the exudate proteome in response to Pi starvation GO terms were used to get an overall idea of the proteins changed in abundance. The significantly DAP in the exudates were analysed for GO term enrichment using PANTHER 16.0, to determine whether GO terms were significantly enriched in Pi starved exudates using a Fisher exact test with *Arabidopsis thaliana* as a background reference and FDR value set at 0.05. Regarding the biological process very few terms were enriched in the proteins that increased in abundance in Pi starved exudates, only terms related to carbohydrate metabolic process, and guard cell development were enriched. While the proteins that were decreased in abundance in Pi starved exudates had various enriched terms, with the highest FE including glycerol catabolic process, gluconeogenesis, ribosomal assembly and translation (Fig.3.5C). This was not surprising considering that a large proportion of proteins less abundant in the Pi starved exudates were considered to be ribosomal related; 25 out of 71 proteins (35.2%), these proteins do not contain a SP and it is unlikely that they are actually secreted from the roots. But as the control samples are grown in the same growth conditions (aside from Pi availability) as the Pi starved samples then I think it is worth mentioning their presence in the control exudates compared to the Pi starved exudates but they are not included in the protein Table 3.1. These results show that the exudate proteome has significantly changed biological processes in response to Pi starvation.

The Molecular function GO terms showed that the exudate proteins that increased in abundance in response to Pi starvation resulted in an enrichment in a variety of terms that could be linked to Pi stress conditions, such as acid phosphatase activity, NADH pyrophosphatase activity, glucosidase activity and GDPD activity

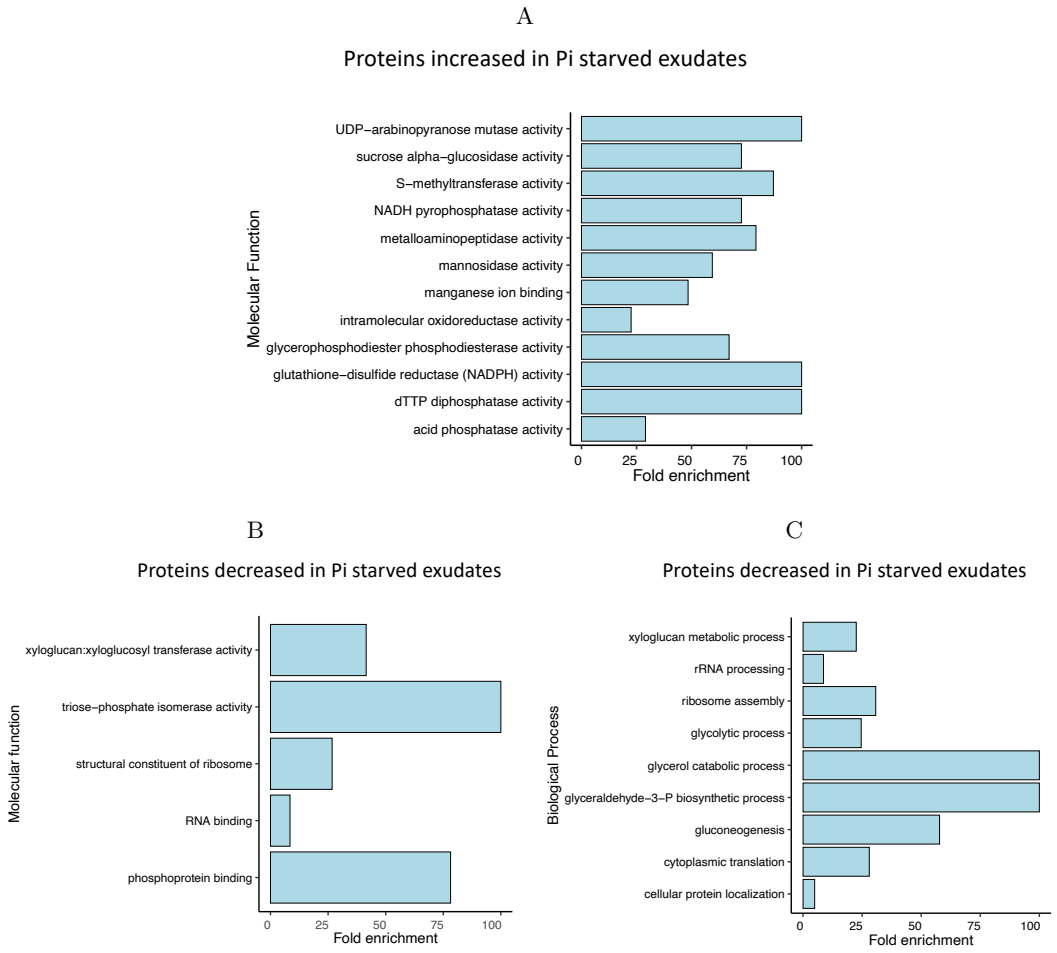

Figure 3.5: ***Arabidopsis*** **exudate proteome GO-Term enrichment:** GO term analysis of the significantly differentially abundant exudate proteins using PANTHER16.0. Displaying the terms FE which are considered significantly enriched based off a Fisher exact test with FDR < 0.05. A) Molecular Function GO term analysis of proteins increased in abundance in Pi starved exudates. B) Molecular Function GO term analysis of proteins decreased in abundance in Pi starved exudates. C) Biological Process GO term analysis of proteins decreased in abundance in Pi starved exudates.

(Fig.3.5A). The proteins which decreased in abundance in Pi starved exudates had fewer enriched molecular function terms, these included phosphoprotein binding, phosphate isomerase activity and structural constituent of ribosomes (Fig.3.5B), which matches with the biological process enrichment of ribosomal assembly.

To get an idea of the different types of proteins which were identified within the exudates and whether they changed significantly, PANTHER16.0 was used to identify the Panther Protein Classification of the proteins that change significantly in response to Pi starvation. An enrichment analysis on the protein classes was also performed to see if any classes are significantly altered in response to Pi starvation.

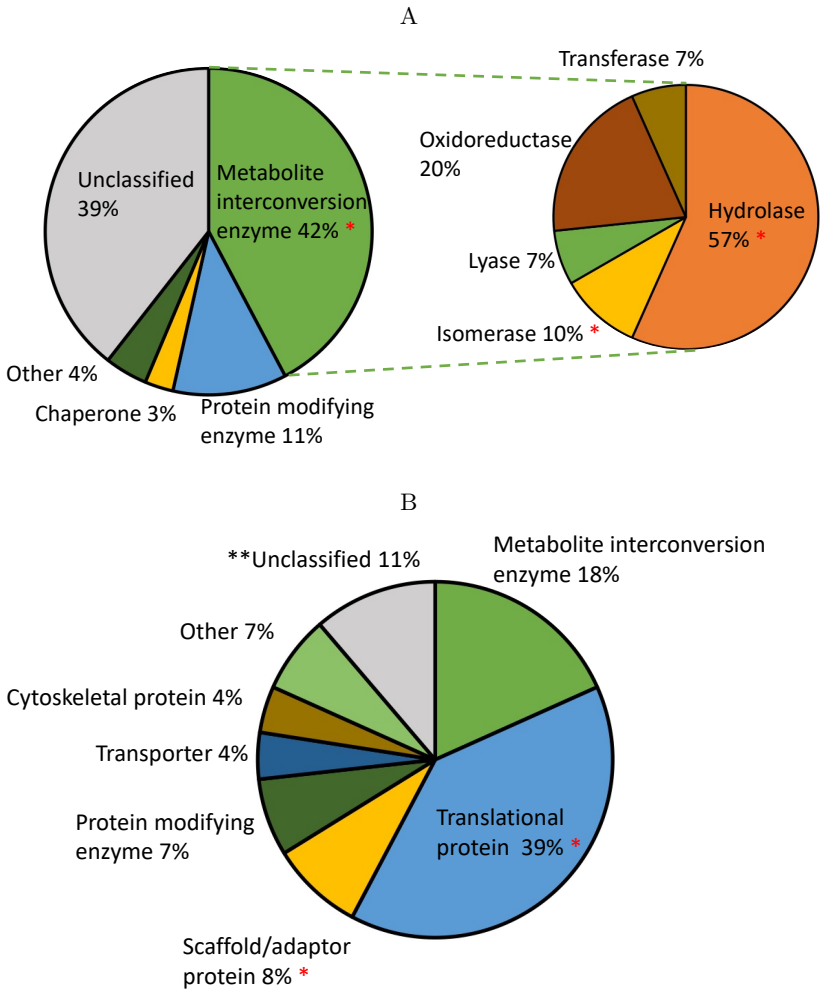

Figure 3.6: Classification of *Arabidopsis* exudate proteome: The exudate proteins that changed significantly in abundance Protein Classifications were determined using PANTHER 16.0. and enrichment analysis of the PC was also determined. A) The proteins that increased in abundance in Pi starved exudates PC, with the split of the metabolite interconversion enzymes also shown. B) The proteins decreased in abundance in Pi starved exudates PC. * Denotes PC which were significantly over enriched, ** Denotes PC which were significantly under enriched using Fishers Exact test with a FDR < 0.05 and *Arabidopsis* as a background reference.

The proteins increased in abundance in Pi starved exudates had the largest proportion of proteins classed as metabolite interconversion enzymes at 42% (Fig. 3.6B), which was significantly enriched, these enzymes include an enrichment for hydrolase and isomerase proteins. The proteins that were decreased in abundance in Pi starved exudates showed that the majority of the PC identified were Translational proteins at 39% (Fig. 3.6A) which was significantly enriched and includes ribosomal proteins, scaffold/adaptor proteins were also enriched. The PC of the exudate proteins indicates that there is a significant change in the *Arabidopsis* exudate proteome in

response to Pi starvation.

All of these results indicate a drastic change in Col-0 exudate proteome in response to Pi starvation. The predicted classification, functions and processes of these changed exudate proteins significantly change. Proteins which increase in abundance include PAP and RNase proteins, which could be indicative of a change of *Arabidopsis* exudates to help improve the Pi efficiency of Col-0 plants during Pi starvation to help the plants adapt to the low Pi availability.

3.2.3 PSR in *Arabidopsis* leads to an altered phosphoproteome indicating a change in signalling pathways

Signalling events are expected to change in response to Pi availability as a part of *Arabidopsis* PSR. Phosphoproteomics can be used to help identify some signalling events mediated by phosphorylation, as this PTM is commonly used in signalling pathways in plants. To identify proteins that have changed phosphorylation status, phosphopeptide enrichment was performed. Whole protein extracts from 10 day old *Arabidopsis* seedlings either starved of Pi for 5 days, or maintained in control conditions, were extracted, with 3 replicates for each condition. Proteins were digested using FASP digest and the resultant peptides underwent phosphopeptide enrichment using TiO_2 beads then identified using MS.

After the MS analysis a total of 25,159 MS spectra were recorded which were assigned to 5,116 peptides. These peptides were then filtered by the removal of low confidence hits, anomalous peptides, potential contaminants, and reverse values, localisation score of at least 0.75, and further filtered so that the phosphopeptide had to be identified in all three replicates of at least one Pi condition to be considered confidently present, this left 1,054 phosphopeptides assigned to 713 protein groups identified for further analysis.

Using the LFQ intensity values these phosphopeptides were further analysed to determine changes in phosphoproteome in response to Pi starvation. A PCA was performed (Fig. 3.7A) and shows a distinct separation of the Pi starved phosphoproteome compared to control phosphoproteome, with a component 1 value of 38.5%, indicating a difference in the phosphoproteome of *Arabidopsis* seedlings in response to Pi starvation.

A standard two-sample t-test was performed on the phosphopeptides $\log_2$ LFQ intensities to determine significantly differentially abundant phosphopeptides in response to Pi starvation, using a FDR value of 0.05 and s0 value of 0.1. A volcano

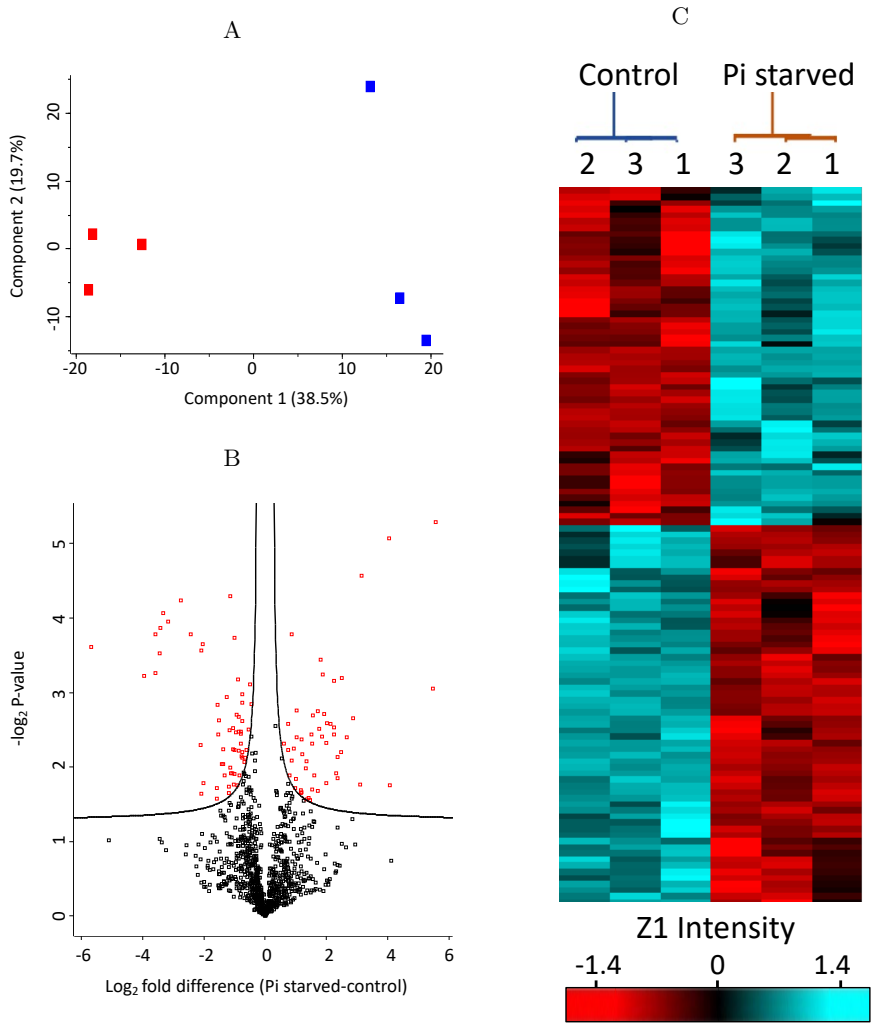

Figure 3.7: Quantitative analysis of *Arabidopsis* phosphoproteome in response to Pi starvation: The phosphopeptides identified in the *Arabidopsis* seedlings were analysed in Perseus using the LFQ intensity values. A) PCA plot of phosphopeptides, Pi starved phosphoproteome samples highlighted in red, and control phosphoproteome samples are highlighted in blue. B) Volcano plot of phosphopeptides, using a student two-sample t-test to identify the phosphopeptides significantly changed in abundance between conditions, $\log_2$ fold change is plotted against -logP value. Red points show phosphopeptides identified which are significantly changed in abundance in Pi starved seedlings. FDR: 0.05 s0: 0.1. C) Heat map of the 118 significantly changed phosphopeptides in response to Pi starvation. LFQ $\log_2$ intensity values normalised using Z-score for graphical representation. There were three replicates for each conditions.

plot was used to represent this data, plotting the $\log_2$ FC difference of Pi starved intensities minus the control LFQ intensities against the -log p-value (Fig. 3.7B), points in red indicate the phosphopeptides significantly changed in abundance. Of the 1,054 phosphopeptides identified 117 were considered to be significantly differentially abundant in response to Pi starvation, these were assigned to 102 different proteins. 55 are significantly more abundant in Pi starved phosphoproteome while 62 are significantly less abundant in Pi starved phosphoproteome. The phosphopep-

tides determined to be differentially abundant $\log_2$ LFQ intensities were normalised using z-score then used to create a heat map (Fig. 3.7C), the samples clustered clearly based on the Pi conditions with a clear distinction between the phosphopeptides more or less abundant in response to Pi starvation. These results show that there is a significant difference in the phosphoproteome of Col-0 seedlings during phosphate starvation compared to Col-0 seedlings maintained in sufficient Pi conditions.

A subset of phosphopeptides which significantly increased in abundance in response to Pi starvation are shown in Table 3.2. The proteins with phosphopeptides increased in abundance in response to Pi starvation had an enrichment in biological processes; cellular response to Pi starvation which were also shared by an enrichment in galactolipid biosynthetic process, and sucrose biosynthetic process, the phosphoproteins and peptides identified involved in these processes are indicated in Table 3.2, there was also an enrichment in the molecular function sucrose-phosphate synthase.

A subset of phosphopeptides which are significantly less abundant in the Pi starved phosphoproteome by more than a $\log_2$ fold change of 2 are shown in Table 3.3, this was to make the table size more manageable to represent the changes in the phosphoproteome. The proteins with phosphopeptides that decreased in abundance in response to Pi starvation did not have any GO term functions or processes enriched.

Table 3.2: *Arabidopsis* phosphopeptides increased in abundance in response to Pi starvation: A subset of the phosphopeptides identified that are significantly increased in abundance Pi starved Col-0 phosphoproteome, and grouped in biological process. FC; $\log_2$ fold change of the peptides, and -log p-value of the FC, MQ score of detected peptide. Protein ID's and names from uniprot. Sequence window which has the phospho modification identified in bold. Site states the position within the protein which was phosphorylated.

Protein ID	Protein name	Sequence window	Score	$\log_2$ FC	-log P value	Site
Cellular response to Pi starvation						
AT4G00550	Digalactosyldiacylglycerol synthase 2	LNTP**S**PNTR	133.5	2.1	2.6	S466
AT3G14940	PEPC 3	EKMA**S**IDAQ	119.2	1.9	3.2	S11
AT1G53310	PEPC 1	EKMA**S**IDVH	102.5	1.7	2.8	S11
AT3G09560	Phosphatidate phosphatase PAH1	DDPP**S**PTSE	105.0	1.3	2.0	S162
AT3G05630	Phospholipase D zeta 2	ARHD**S**FSSA	83.8	1.2	2.4	S629
Sucrose biosynthetic Process						
AT4G10120	Probable sucrose-phosphate synthase 4	LTSD**S**LRDV	152.7	2.7	2.4	S717
		LSEL**S**EGEK		2.3	1.9	S148

Continued on next page

Table 3.2 – *Continued from previous page*

Protein ID	Protein name	Sequence window	Score	log_2 FC	-log P value	Site
		PRIR**S**EMQI		1.3	1.6	S180
AT5G20280	Sucrose-phosphate synthase 1	SEPE**S**PSDS	160.1	2.5	2.2	S688
AT5G11110	Probable sucrose-phosphate synthase 2	INDI**S**LNLK	95.6	2.0	2.3	S701
Misc						
AT4G13350	NSP -interacting GTPase	LGSA**S**PPVA	77.1	5.6	5.3	S219
AT2G17980	SEC1 family transport protein SLY1	ANSA**S**RSNI	88.1	5.5	3.1	S480
AT1G68370	Chaperone protein dnaJ 15	M**S**AKKL	146.8	4.1	1.8	S2
AT1G80530	Major facilitator superfamily protein	NAKS**S**PLGS	66.1	4.1	5.1	S272
AT5G15680	ARM repeat superfamily protein	PFGY**S**PADP	82.8	3.1	4.6	S1075
AT5G42860	Late embryogenesis abundant protein	VQSP**S**RDSH	96.3	3.1	1.8	S32
AT3G13530	MAP3K epsilon protein kinase 1	SGIA**S**QTAS	40.52	2.9	2.7	S1005
		SGVL**S**GSGV	40.52	2.7	2.4	S1013
AT5G17010	D-xylose-proton symporter-like 2	SGEI**S**PERE	155.1	2.5	3.2	S26
AT4G31880	Transcriptional regulator	SLHT**S**SGDN	96.3	2.4	2.1	S558
AT5G57610	Kinase superfamily	QYPD**S**PRFN	147.3	2.4	1.9	S169
		KTLV**S**GGVR	100.3	2.3	3.2	S945
		PRVG**S**GQML	95.5	1.0	2.4	S220
AT2G23520	Pyridoxal phosphate (PLP)-dependent transferases	HKIA**S**PLPP	90.1	2.2	2.4	S530
AT5G52640	Heat shock protein 90-1	EKEI**S**DDED	198.6	2.0	2.6	S219
AT1G60940	Serine/threonine-protein kinase SnRK2	SLLH**S**MPKS	88.1	1.2	1.7	S154

Table 3.3: *Arabidopsis* phosphopeptides decreased in abundance in response to Pi starvation: A subset of the phosphopeptides identified that are significantly decreased in abundance in Pi starved Col-0 phosphoproteome. FC; log_2 fold change of the peptides, -log p-value of the FC, MQ score of detected peptide. Protein ID's and names from uniprot. Sequence window which has the phospho modification identified in bold. Site states the position within the protein which was phosphorylated..

Protein ID	Protein name	Sequence window	Score	log_2 FC	-log P value	Site
AT1G59610	Dynamin-2B	RQSL**S**EGSL	99.5	5.7	3.6	S719
AT5G53170	ATP-dependent zinc metalloprotease	KHRA**S**GNMD	41.4	3.9	3.2	S264
		NMDE**S**FVNP		3.6	3.3	S270
		NPGI**S**EKQP		3.4	3.9	S277
AT4G36860	LIM domain-containing protein	MNVG**S**PPRY	82.6	3.6	3.8	S166
AT3G28180	Xyloglucan glycosyltransferase 4	TGRS**S**ESDL	138.2	3.5	3.5	S562
AT2G20190	CLIP-associated protein	ALRS**S**SLDL	86.5	3.3	4.1	S612
AT5G58140	Phototropin 2	RAPP**S**PLND	113.4	3.2	4	S9
AT5G08050	Uncharacterized protein	FSLS**T**IERL	177.6	2.8	4.2	T73
AT3G63500	Fibronectin type III domain protein	LFSS**S**PVRR	126.7	2.4	3.8	S197
AT4G09320	Nucleoside diphosphate kinase 1	VIHG**S**DSVE	113.4	2.1	2.3	S117
AT5G36880	Acetyl-coenzyme A synthetase	MKIG**S**PSSP	74.9	2.1	3.6	S5
AT5G19010	Mitogen-activated protein kinase 16	FWTD**Y**VATR	145.5	2.1	1.6	Y189
AT3G45780	Phototropin-1	SGEM**S**DGDV	136.3	2.0	3.6	S170
AT3G15450	MJK13.11	PRRG**S**EANW	120.6	2.0	1.8	S243
AT5G65690	PEPCK (ATP) 2	QKKR**S**APTT	96.0	2.7	-0.9	S66

The majority of the phosphopeptides identified within the *Arabidopsis* phosphoproteome were mono phosphorylated at 87.8%, 8.6% of the peptides carried 2 phosphosites, and 3.6% of the peptides had three phosphopeptide sites. The most common phosphorylated amino acid was phosphoserine with 89.9% of the phosphosites being on a serine residue, 9.2% of the modifications were phosphothreonine, and 0.9% were phosphotyrosine.

Phosphosites can often contain motifs which are common patterns around the modification site. MoMo motif-x analysis can be used to identify the commonly occuring phosphomotifs and match them against a background proteome to see if there are motifs that are enriched in a dataset. MoMo motif-x analysis on all 1,054 phosphopeptides detected in the *Arabidopsis* phosphoproteome showed that there were 13 motifs that were significant in the dataset (Table. 3.4) [Cheng et al., 2019]. Of the phosphopeptides that were significantly changed in the phosphoproteome in re-

sponse to Pi starvation, only the motif -Sp- was significantly enriched, in both the phosphopeptides increased and decreased in response to Pi starvation.

Table 3.4: Motif analysis of *Arabidopsis* phosphoproteome: Phosphopeptides detected in *Arabidopsis* seedlings went through MoMo motif-x analysis to determine enriched motifs, with a P-value threshold of $< 1e^{-}6$, using *Arabidopsis* background [Cheng et al., 2019].

Motif	Foreground matches	Background matches	Score
.....SP.....	477	65218	78
...R..S.....	224	68747	52
....P.SP.....	93	5340	179
...R..SP.....	77	4199	331
.....S..E...	75	81094	8
...K..S.....	74	70983	10
.....SP.R...	73	3860	275
.....S..D...	72	66990	7
.....SPR....	68	4290	140
.....RSP.....	62	3322	209
.....TP.....	51	34994	39
.....SPK....	46	3376	110
...R..SF.....	26	2880	78
....P.SPR....	22	321	361

The results for the phosphoproteome analysis indicate a vast change in the phosphoproteme of *Arabidopsis* seedlings in response to Pi starvation. Phosphoproteome changes can occur in response to Pi treatments, due to a change in signalling events that occur in response to Pi starvation.

The number of proteins that are shared between the exudate proteome and phosphoproteome is minimal with only 23 being identified in both datasets. When looking at the proteins which changed significantly in response to Pi starvation, there were none which overlapped between the exudate proteome and phosphoproteome.

3.3 Discussion

The *Arabidopsis* PSR phenotypes were as expected based on the literature; seedling growth was inhibited when grown in Pi deficient conditions compared to Pi sufficient conditions, shown by seedlings fresh weight, and there was a decrease in primary root length which is a known PSR [Lynch and Brown, 2001]. There was also an increase in acid phosphatase activity on the root surface as well as in the root exudate proteins and seedling protein extracts of Pi starved Col-0.

An increase in the acid phosphatase activity of membrane associated and secreated proteins had previously being reported in *Arabidopsis* plants in response to Pi starvation [Jiang and Hong, 2015, Wang et al., 2011a]. These adaptations can be suggested to be for the improvement of the accessibility of Pi to be taken up by *Arabidopsis*. The PAP proteins function to release Pi from Po sources therefore improving the availability of accessible Pi. There was an increase in abundance of PAPs detected in the exudate proteome with an enrichment for molecular function GO term acid phosphatase activity in response to Pi starvation. Three different PAPs (AtPAP26, AtPAP1, AtPAP17) were identified to be significantly increased in abundance in the Pi starved *Arabidopsis* exudate proteome, which correlates with the increase in activity also observed, which was encoraging to see as an increase in protein abundance does not always mean an increase in activity.

Previous work by Wang et al. [2014a] on secreated *Arabidopsis* APase proteins have shown that AtPAP10, 12, and 26 are three commonly secreated PAPs in response to Pi starvation. They have also shown with in-gel APase activity assays that the APase activity at approximetly 80 KDa is caused by AtPAP26. Figure 3.2E shows that the exudate proteins at approximatly this same molecular weight (80 KDa) have increased APase activity in Pi starved exudates in my in-gel APase activity assay. AtPAP26 was also identified by MS to increase in abundance by $\log_2$ three fold change in the Pi starved exudates (Table 3.1). This shows a correlation between the abundance of AtPAP26 and an increase in APase ativity in the Pi starved exudate proteome, though I can not directly show a causal relationship. AtPAP26 has also been documented to have an increase in expression in *Arabidopsis* suspension cells when grown in Pi deficient conditions, as well as being root secreted when plants are grown hydroponically [Tran et al., 2010, Veljanovski et al., 2006], it was also shown that AtPAP26 is dual targetted and is also functional as an intracellular vacuole PAP that gets induced during low Pi conditions, allowing an increased release of available Pi from intracellular storage P to be made accessible to the plant to be utilised.

There were two alkaline phosphatase proteins that increased in abundance in *Arabidopsis* exudate proteome in response to Pi starvation, these proteins have not been identified before to be responsive to Pi starvation in previous studies to the best of my knowledge. However some pyrophosphatase proteins have been considered to be involved in PSR, these pyrophosphatase proteins are a part of the family of alkaline phosphatase proteins [May et al., 2011]. The enrichment in dTTP diphosphatase activity and NADH pyrophosphatase activity in Pi starved exudates (Fig. 3.5A) can

be attributed to by the increased abundance of the alkaline phosphatases. dTTP diphosphatase activity catalises the release of diphosphate and dTMP from dTTP which could potentially allow the realease of Pi to be utilised by the plants and NADH pyrophosphatase activity is part of the nucleotide diphosphatase activity hierarchy. These could be involved in the breakdown of nucleic acids which are a source of Pi.

Ribonuclease 1 (AT2G02990) was increased in abundance in the Pi starved exudate proteome. RNase proteins have been thought to be secreated to improve the accessibility of Pi for plants to utilise. This induction of RNase had been detected in tomato plants in response the Pi stress [Abel et al., 2000]. Nucleic acids are a bountiful source of phosphate with approximately 9% of RNA dry weight taken up by phosphate [Raven, 2013], so the degradation of extracellular RNA substrates using RNase proteins to improve Pi availability is a mechanism plants could utilise when in low Pi conditions to scavenge for exogenous phosphate [Nürnberger et al., 1990]. The extracellular RNase 1 identified in the tomato cultures is a homologue of the RNase P42813 identified to have increased in abundance in the Col-0 exudates, it is also the same RNase identified to be increased in abundance in *Arabidopsis* suspension cells in response to Pi starvation by Tran and Plaxton [2008].

There are a number of proteins involved in carbohydrate metabolic processes that are increased in abundance in Pi starved exudates, and is an enriched biological process in the Pi starved exudate proteome. The specific roles these proteins play in response to Pi deficiency to imporve Pi efficiency is unclear.

Germin-like protein (GLP) family were significantly more abundant in the Pi starved exudate proteome, 4 increased in abundance and also contain a SP. These proteins are soluble glycoproteins and play a role in plant responses to biotic and abiotic stresses, they are more commonly reported on their response in plants against pathogens [Pei et al., 2019], but they can have a variety of enzymatic functions which can be associated with a response to phosphate stress, such as defence/detoxyfying, potentially signaling via superoxide dismutase activity [Zhang et al., 2017], and pyrophosphatase/phosphodiesterase activity [Fan et al., 2005, Rodríguez-López et al., 2001]. Further analysis on the GLPs identified will need to be completed in order to see what the specific functions are, and could offer an interesting insight into some abiotic responses of GLP proteins in response to Pi starvation.

The phospholipid bilayer which makes up cell membranes are another rich source of phosphate, the breakdown of the cell membranes can then provide a release of

phosphate that plants could scavenge, two GDPDL proteins were significantly more abundant in the exudate proteome in response to Pi starvation. These proteins catalyse the reaction of glycerophosphodiester plus water to produce an alcohol and sn-glycerol 3-phosphate (G3P), therefore GDPD catalyses the break down of lipids to release G3P, which even though it is not a functional nutrient form of phosphate it is suspected to be important for Pi homeostasis in plant cells, or it could be further broken down by phosphatases to release Pi, and can be used as a precurser for the production of non phosphate containing galactolipids to replace the phospholipids [Ramaiah et al., 2011].

The detected phosphopeptides that increased in abundance in *Arabidopsis* seedlings had an enrichment of proteins that were involved in galactolipid biosynthesis which is a cellular response to Pi starvation, which links in with the exudates increased abundance of GDPD proteins in response to Pi starvation. Phosphatidate phosphatase PAH1 has an increased abundance of phosphorylation at S162, this modification had been identified before and is documented on PhosPhAt (A specalist plant phosphorylation database) but not in response to Pi starvation and the resultant effect the modification has on the protein in unknown. PAH1 is a part of galactolipid biosynthesis and represes phospholipid biosynthesis [Eastmond et al., 2010]. Phospholipase D (AT3G05630) is also involved in the breakdown of phospholipids and in response to Pi starvation contributes to improved Pi availabilty [Li et al., 2006]. A phosphorylation at S629 is increased in abundance detected in Pi starved *Arabidopsis*, this modification had not been documented previously and the effect of the phosphorylation is not known. Digalactosyldiacylglycerol (DGDG) synthase 2 (AT4G00550) contributes to biosynthesis of non P containing galactolipid DGDG, has an increased abundance of phosphorylation at S466, which has been seen before in response to drought stress in PhosPhAt but has not been documented in response to Pi stress. The abundance of proteins involved in lipid remodelling identified in response to Pi starvation indicates that lipid remodelling is an induced PSR.

Identifying phosphosites in proteins during Pi starvation that are not phosphorylated or that are less abundant in control conditions (or vice versa) can give an insight into some of the signalling events that change and take place during Pi starvation. Multiple events within cells are regulated by phosphorylation such as signalling, secreation, and protein activation. The interpretation of phosphosite changes needs to be taken carefully as the overall changes in abundance of proteins can effect the results as well as changes to phosphosites in close proximity can effect the detection of responses.

PEPC proteins are expected to have three roles in plant adaptation to low Pi conditions; through the production of organic acids ready for secretion, it's a part of a glycolytic bypass to the ADP-limited cytosolic pyryvate kinase, and thirdly through the recycling of Pi from phosphoenolpyruvate (PEP). PEPC bypasses pyruvate kinase during glycolysis to facilitate continued pyruvate supply while recycling Pi for its use in metabolism of Pi deficient cells. PEPC catalyses the carboxylation of PEP to yield oxaloacetate and Pi. Phosphorylation at a conserved N-terminal serine residue S11 is catalysed by PEPCK (PEPC kinase). Phosphorylation typically modulates PEPC sensitivity to allosteric effectors by relieving its inhibition by malate while enhancing activation by hexose-phosphates [Bowyer and Leegood, 1997, Gregory et al., 2009, Wu et al., 2018]. PEPC1 and PEPC3 were found to be phosphorylated at S11, to a significantly more abundant amount in response to Pi starvation which has previously been documented to occur in *Arabidopsis* [Gregory et al., 2009] and is considered reversibly phosphorylated in response to Pi deprivation resulting in an increased activity.

A kinase protein will catalise the transfer of a phosphate and are vital in signal transduction, a number of kinases were identified in the phosphoproteome to change in response to Pi starvation. These could provide some insights into the signalling players of Pi starved *Arabidopsis* seedlings.

Motif analysis of phosphosites can provide insights into the kinases that target those motifs. The motif analysis of phosphosites detected to both increase and decrease in reponse to Pi starvation showed that -sp- motif was enriched. This motif is a common occurrence within phosphomodified proteins and are targets of multiple kinases such as MAPK proteins, SnRK2, and cystine rich receptor like kinases van Wijk et al. [2014]

Mitogen-activated protein kinase (MAPK) are a conserved family of kinases, that are specific to the phosphorylation of threonine or serine amino acid residues, they are often involved in signal cascades where a MAPK kinase kinase (MAPKKK) activate MAPK kinase (MAPKK), which in turn activate MAPK, they are implicated in the signalling of many enviromental stresses and plant development processes [Mizoguchi et al., 1997][Xu and Zhang, 2015]. In *Arabidopsis* there are 20 MAPK, 10 MAPKK, and 60 MAPKKK predicted [MAPK Group, 2002]. The activation of MAPKs by phosphoryltion occurs in the TXY motif by activated upstream MAPKKs [Cobb and Goldsmith, 1995].

Some MAPK proteins show a change in phosphorylation status in response to Pi

starvation in the *Arabidopsis* phosphoproteome. In response to Pi stress *Arabidopsis* had previously seen activation of MAPK3 and MAPK6 by Lei et al. [2014], The activation of these MAPK showed to induce expression of Pi acquisition-related genes and enhanced Pi uptake and accumulation, However a rice study on 4 week old plants starved of Pi for seven days, showed an opposite reaction, the dephosphorylation of MAPK6 in response to Pi deficiency [Lei et al., 2014, Yang et al., 2019] indicating a difference in reponse and signalling to Pi deficiency in the different plant species. MAPK 16 was identified to have decreased in phosphorylation in its TDY motif in response to Pi starvation in the Col-0 phosphoproteome (Table 3.3), while MAP3K had an increased phosphorylation in response to Pi starvation (Table 3.2).

Another type of kinase identified was serine/threonine-protein kinase which is part of the SnRK2 family, was phosphorylated in response to Pi starvation, a homologue of this protein in rice was reported to be dephosphorylated in response to Pi starvation [Yang et al., 2019], but not in the same equivelent amino acid residue. *Arabidopsis* SnRK2 was phosphorylated at the S153 residue which has been documented before by Jones et al. [2009] but not in response to Pi stress. The difference between rice and *Arabidopsis* could potentially indicate another difference in PSR between species.

The GO term for sucrose biosynthetic process was enriched in the proteins which had increased phosphopeptides identified in Pi starved phosphoproteome, these included thress sucrose phosphate synthase proteins (SPS1, 2, and 4). These are key enzymes involved in sugar metabolic pathways in plants, and the activity is regulated by phosphorylation through calcium dependent kinase [Bilska Kos et al., 2020], sucrose transport and signalling are a part of shoot to root communication in response to Pi starvation.

It has previously been stated in other studies that phosphate transporters undergo various PTMs, and phosphorylation acts to modulate the transporters exit from the ER and its localisation to plasma membranes [Bayle et al., 2011], but no PHT transporters were identified within my *Arabidopsis* phosphoproteome in response to Pi starvation. In the future it might be worth doing a membrane enrichment from the extracted proteins before digest and phosphopeptide enrichment to be better able to identify membrane proteins.

One limitation of phosphoproteomic studies is the inability to differentiate between changes in phosphorylation stoichiometry from changes in protein abundance. Phos-

phorylation stoichiometry is the ratio of the amount of protein phosphorylated on a particular site to the amount of protein. There is, unsurprisingly, very little overlap of proteins identified between the whole seedlings phosphoproteome and the exudate proteome, of the 499 exudate proteins and 712 phosphoproteins identified only 23 are identified in both samples, therefore the proteomes are not comparable.

3.4 Conclusion

Arabidopsis exudate proteome and phosphoproteome change significantly in response to Pi starvation. There is an increase APase acitivity as well as an increase in abundance of APase proteins secreted from *Arabidopsis* seedlings in response to Pi starvation. The increased in abundance of proteins like PAP and RNase proteins in the exudates in response to Pi starvation likely contribute to improving Pi accessibility and uptake efficiency during low Pi availability, but further work would be needed to confirm protein activity and the effect on Pi efficiency. Signalling events within *Arabidopsis* seedlings appear to change as a part of a phosphate starvation response, as indicated by the changes in the phosphoproteome detected in Pi starved seedlings, but further work would need to be done to understand and piece together the signalling pathways involving protein phosphorylation during Pi starvation.

Chapter 4

Quantitative Proteomics of Phosphate Starved *Brassica rapa* Exudates

4.1 Introduction

Brassicas are globally a highly important crop species, with *B. rapa* consisting of several varieties of well known crops, including turnips, chinese cabbage and yellow sarson. Despite the importance of these crops there has not been as much research documented on their molecular responses to insufficient Pi conditions, and their not as well characterised as other species like *Arabidopsis*. Therefore, I want to see how *B. rapa* will respond to insufficient Pi conditions, using two *B. rapa* lines; the P-efficient R500 yellow sarson and the P-inefficient highly inbred IMB211 rapid cycling line. Comparing the phosphate starvation response (PSR) of the lines which are considered to have different phosphate efficiency's could give some insight into what occurs within the plants to give them greater tolerance to Pi deficiency so that they are better able to adapt to the changes in their environment. Various measures of P-efficiency can be used to describe a plants Pi efficency; Agronomic P use efficiency, measures increase in plant yield per added unit of P fertiliser. P uptake efficiency, measures the increase in plant P content per unit of added P fertiliser. P utilisation efficiency, measures the increase in plant yield per increase in plant P content [Hammond et al., 2009]. R500 was determined to have higher Pi efficiency in all these P efficiency measurements compared to IMB211 by Hammond

et al. [2011]. Another study on R500 and IMB211 PSR by Adu et al. [2017] showed that the number of LR was not affected by Pi availability in either R500 or IMB211 and only R500 displayed a slight decrease in PRL.

A mapping population was developed using the R500 and IMB211 lines called BraIRRi by Iniguez-Luy et al. [2009], this mapping population was used for expression quantitative trait locus (QTL) analysis by John Hammond et al. [2011] in response to low Pi availability, this identified P responsive transcripts enriched with P metabolism-related GO terms and helped to determine that the R500 line was P-efficient while IMB211 is considered P-inefficient line, which is why these two lines were chosen to give a comparison for *B. rapa* Pi efficiency in this chapter.

A QTL is a section within chromosomes that is linked to certain traits within the species population, for example in the context of this work these traits can include phenotypic responses to phosphate stress, so the mapping performed allows these loci to be identified which correlate to a plants response to Pi stress conditions [Miles and Wayne, 2008]. In theory QTL analysis allows the phenotypes observed within plants to be matched with specific areas of a chromosome, or within certain genes, although it is not as simple as that, as QTL areas can be huge and interactions of multiple genes, PTM and localisation of the gene products also play a complex role in the outward appearance and response of a plant, but it is a good start to narrow down the focus of a topic. My aim is to determine at how the gene products change in response to Pi starvation. I want to see how the secreted exudate proteins change and how these changes could be indicative of improved Pi efficiency. The root secreted proteins in response to Pi starvation have not been researched before for *B. rapa* and follows on from the previous chapter where I analysed the changes in *Arabidopsis* exudate proteome in response to Pi starvation, a species like *B. rapa* may not respond in the same way as *Arabidopsis* to Pi starvation making the work novel and interesting.

4.1.1 Aims

I hypothesize that the P-efficient R500 and the P-inefficient IMB211 will respond differently in response to Pi starvation, with R500 inducing a more obvious PSR to adapt to the insufficient Pi conditions, and that the root exudates of these lines will change in response to Pi starvation in such a way to facilitate a higher P efficiency in R500. Identifying the exudate proteins that change will be useful in determining targets that can be used to help to improve a plants tolerance to Pi starvation.

The aims of this chapter were to identify *Brassica rapa* exudates in phosphate efficient and inefficient lines R500 and IMB211 respectively, and to identify changes in exudate proteins in response to Pi starvation, as these changes could be involved in improving the plants phosphate efficiency. To achieve this aim *B. rapa* seedlings were grown hydroponically in either P-sufficient or P-deficient conditions to:

- Induce a PSR in hydroponically grown R500 and IMB211 *B. rapa* lines.
- Measure *B. rapa* physiological responses to Pi starvation.
- Quantitatively analyse the R500 and IMB211 exudate proteomes to determine the differences in PSR.

4.2 Results

In order to analyse the proteomic changes in *B. rapa* root exudates in response to Pi starvation I first needed to establish a PSR. *B. rapa* R500 and IMB211 lines were grown hydroponically until they were 31 days old before they were harvested. Half of the plants had been starved of Pi in Pi deficient media for 11 days to initiate a PSR while the other half were maintained in Pi sufficient media. 24 hours before the plants were harvested they were moved into separate falcon tubes with the roots submerged in their media to collect the root exudates. The root and shoot material was weighed and harvested and the media was collected and freeze dried so the root exudate proteins could be collected for MS analysis (Fig. 4.1).

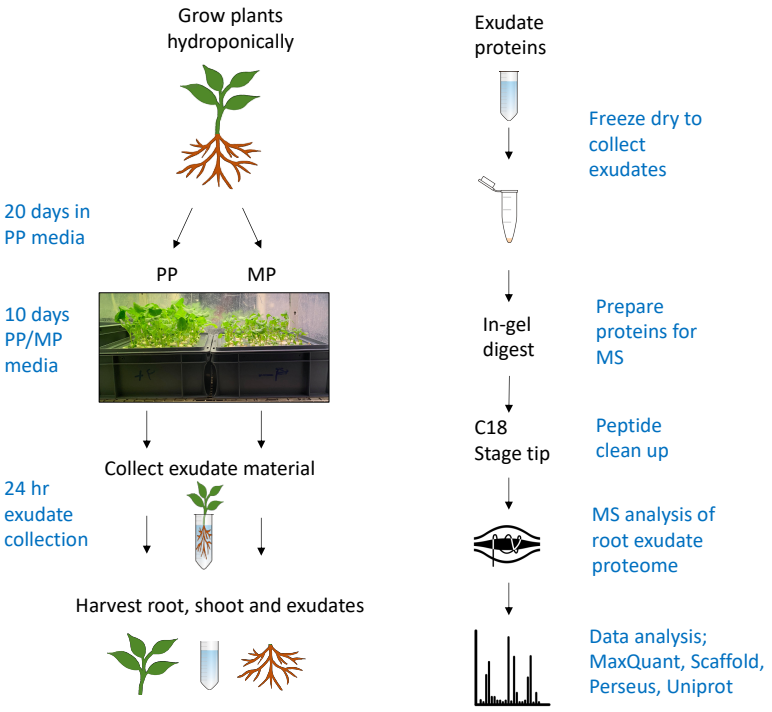

Figure 4.1: Preparation of *B. rapa* root exudate proteome: *B. rapa* R500 and IMB211 lines were grown hydroponically in Pi sufficient (PP) media untill 20 days old, after half were transferred into Pi deficient (MP) media while the other half remained in PP media for a further 10 days. Individual plants were placed into falcon tubes with their roots submerged in their media for 24 hours to collect root exudates, after which the root, shoot and media was of the 31 day old plants were harvested. The media was freeze dried to collect the exudate proteins. Exudate proteins were then prepared for MS by an in gel digest then stage tipped before MS analysis and the data analysed using a variety of software and web tools.

4.2.1 Phosphate starvation response in *B. rapa* leads to physiological changes

Plants will induce a variety of physiological responses to adapt to Pi starvation. A PSR was induced in *B. rapa* R500 and IMB211 plants grown hydroponically until 31 day old, and were starved of Pi for 10 days to measure a variety of physiological responses to Pi starvation.

The shoots of the R500 and IMB211 plants that were Pi starved were visibly smaller and the leaves pigmentation was visually darker compared to the plants that had been grown in control conditions (Fig. 4.2A), the roots and shoots of these plants were then harvested and weighed.

Root fresh weight (FW) was measured before the root material was dried to give the root dry weight (DW) (Fig.4.2C). The R500 plants have a significantly higher FW and DW than the IMB211 plants in both Pi conditions. While the root weights

did not change significantly in response to Pi starvation for either R500 or IMB211 lines (Fig.4.2C).

Shoot FW and DW (Fig. 4.2B). IMB211 have a smaller FW and DW compared to the R500 line regardless of Pi conditions. R500 plants showed a significant decrease in shoot FW and DW in response to Pi starvation. The IMB211 plants had a significant decrease in shoot FW in response to Pi starvation but no significant change in shoot dry weight.

The root to shoot ratio of R500 and IMB211 plants in response to Pi starvation was also measured (Fig. 4.2D), the ratio is mostly the same between the two lines, and increases significantly in response to Pi starvation. This is not surprising as the root material weight stays mostly the same between Pi conditions but the shoot material significantly decreases in response to Pi starvation.

R500 and IMB211 free Pi levels within the shoot material was measured using a malachite green colorimetric assay [Itaya and Ui, 1966, Lu et al., 2016]. The R500 shoots Pi levels were significantly decreased in response to Pi starvation, but the IMB211 shoots did not have a significant change in Pi levels in response to Pi starvation even if there was a decrease (Fig.4.2E).

These changes in the *B. rapa* physiology show a PSR was initiated in the plants deprived of Pi for 10 days compered to plants not deprived of Pi, leading to a change in shoot weight but not a change in the root weight. R500 and IMB211 plants both showed a PSR but the R500 response appeared to be more significant. The root exudate proteins of these plants were used for quantitative proteomic analysis using MS to determine the *B. rapa* exudate proteomes PSR.

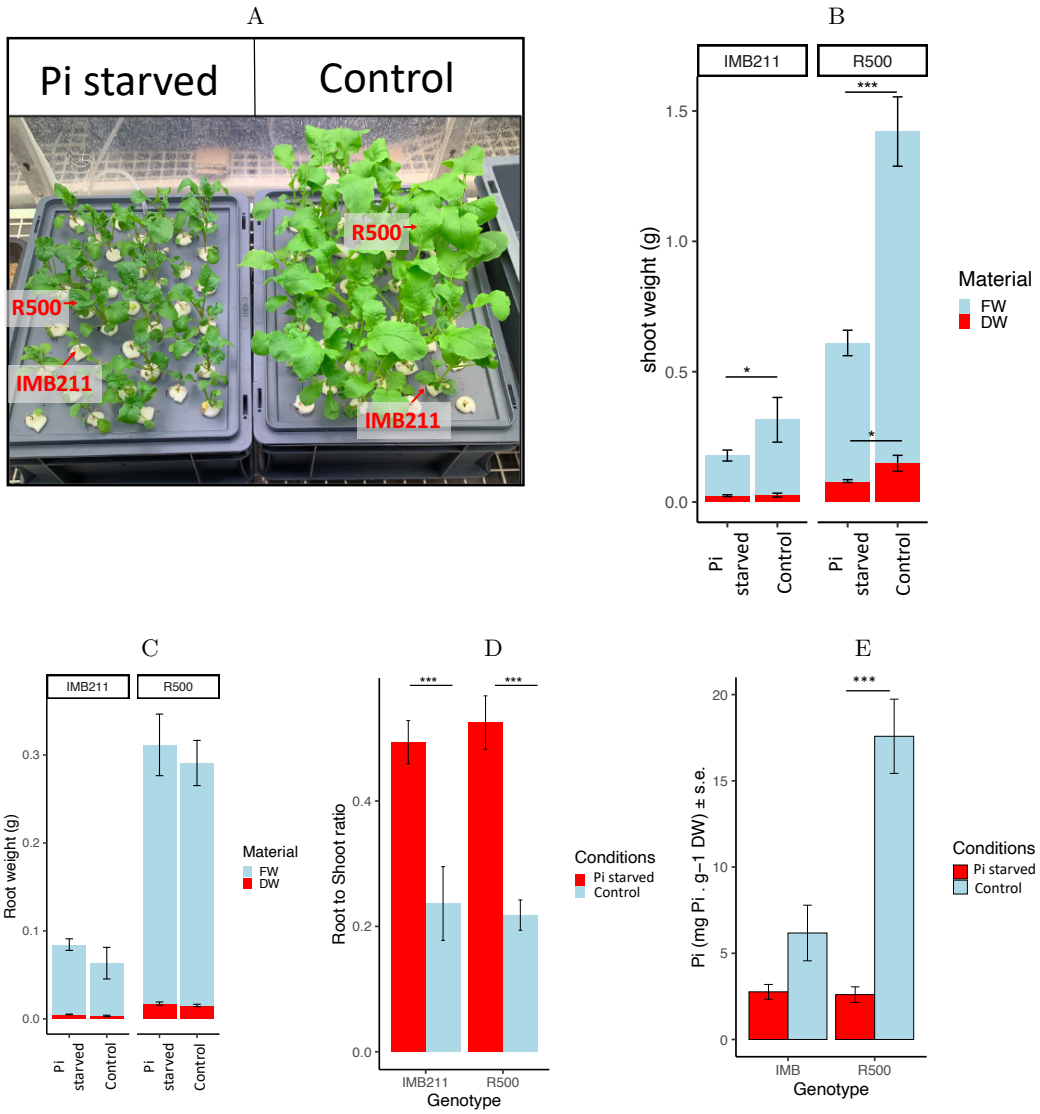

Figure 4.2: Physiological responses of *B. rapa* to Pi starvation: *B. rapa* R500 and IMB211 plants were grown hydroponically until 31 days old with 10 days of Pi starvation treatment before harvest. A) Image of the R500 and IMB211 plants mixed in the hydroponic tanks. B) Shoot FW and DW. C) Root FW and DW. D) Root to shoot ratio of the fresh weight values. E) The Pi levels in the shoots of the plants was determined from the dried material using the malachite green colorimetric assay. Significant difference was determined using a standard two sample t-test; *: $p < 0.05$ ***:$p < 0.0005$, error bars display standard error.

4.2.2 PSR in P-efficient *B. rapa* R500 leads to significant change in exudate protein composition

The *Arabidopsis* exudate proteome in the previous chapter was shown to change significantly in response to Pi starvation, I want to see whether the *B. rapa* R500 exudate proteome also changes in response to Pi starvation. The changes in the

exudates could facilitate the plant to adapt to low Pi availability as a part of its PSR to improve the plants Pi uptake and utilisation efficiency.

The root exudate proteins were collected from *B. rapa* R500 plants grown hydroponically. Exudates were isolated from individual plants over 24 hours after a PSR had been induced, the proteins were identified by MS for quantitative analysis of four replicates.

After the MS analysis 38,343 MS spectra were recorded and assigned to 832 proteins. The proteins were filtered by the removal of low confidence proteins, anomalous proteins, potential contaminants, reverse values, and further filtered so that the protein had to be identified in at least three of the biological repeats to be kept for analysis. One of the control exudate samples was removed from the data set due to it having a low peptide coverage compared to the other samples. After filtering, 593 proteins were identified in the exudate proteome for further analysis.

Using the LFQ intensity values the identified proteins were further analysed to determine any changes in the exudate proteome in response to Pi starvation. A PCA was performed to determine how different the samples were (Fig. 4.3A). The PCA shows a distinct separation of the Pi starved exudates compared to the control samples, with a component 1 value of 53.4%, indicating a clear change in R500 exudate proteomes in response to Pi starvation.

A standard two sample t-test was performed on the LFQ intensities and used to determine the proteins significantly differentially abundant in response to Pi starvation, using an FDR value of 0.05 and s0 value of 0.1. A volcano plot was used to represent this data, $\log_2$ FC difference with Pi starved sample values minus control sample values plotted against -log p-value (Fig. 4.3B), points in red show the significantly DAP. Of the 593 proteins identified 195 were significantly changed, 119 proteins were more abundant in response to Pi starvation, while 76 were less abundant in response to Pi starvation, meaning that 33% of R500 exudate proteome was significantly changed in response to Pi starvation. The proteins determined to be differentially abundant $\log_2$ LFQ intensity levels were normalised using Z-score and used to generate a heat map (Fig. 4.3C). The samples clustered clearly based on the Pi conditions with a clear distinction between the proteins more or less abundant in response to Pi starvation. These results show that there is a significant difference in the exudate proteome of Pi starved *B. rapa* R500 plants compared to R500 plants grown in Pi sufficient conditions.

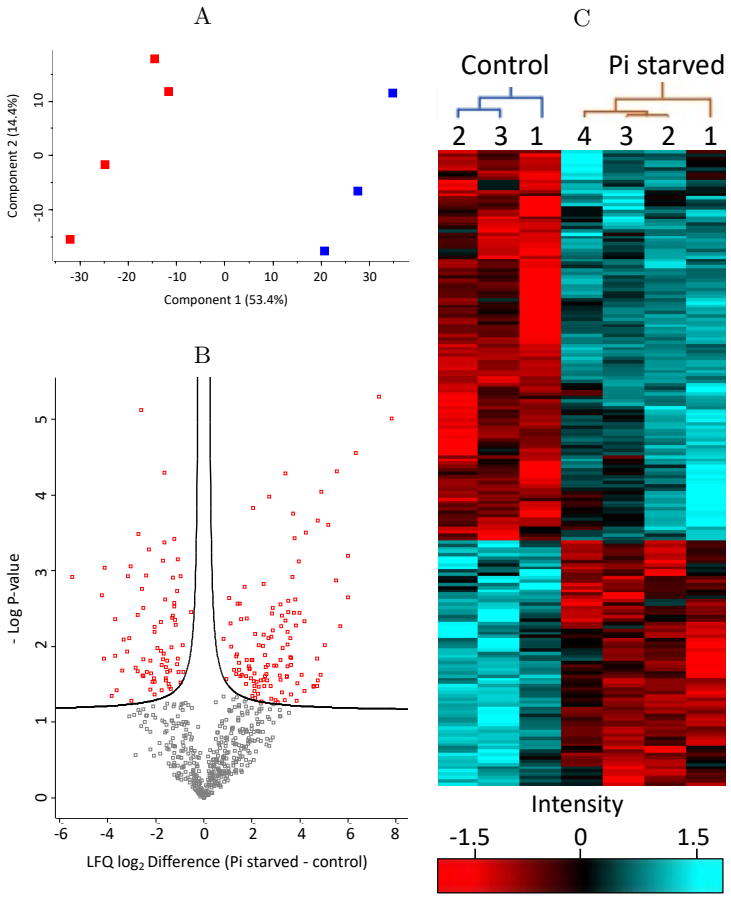

Figure 4.3: Quantitative analysis of *B. rapa* R500 exudate proteome in response to Pi starvation: The proteins identified in the R500 exudate samples were analysed in Perseus using LFQ intensity values. A) PCA plot of exudate proteins. Pi starved exudate samples are highlighted in red, control exudate samples are highlighted in blue. B) Volcano plot of exudate proteins, using a student two sample t-test to identify proteins significantly changed in abundance, $\log_2$ fold change is plotted against -log P-value. Red points show the 195 proteins which are significantly changed in abundance in response to Pi starvation, FDR:0.05 s0:0.1. C) Heat map for the 195 significantly changed proteins during Pi starvation. $\log_2$ LFQ intensity values normalised using Z-score for graphical representation.

4.2.3 P-inefficient IMB211 exudate samples do not show a significant amount or change in protein composition in response to phosphate starvation

The Pi-inefficient IMB211 *B. rapa* exudates were also collected from the plants grown hydroponically after a PSR was induced, and the proteins were identified using MS. After the MS analysis 30,455 spectra was recorded and matched to 258 proteins, these proteins were filtered by removal of low confidence proteins and contaminants, and filtered so that the protein had to be identified in at least 3 replicates of a Pi condition, this left 79 proteins confidently identified. Only 3 of the identified proteins were determined to be significantly changed and more abundant in response to Pi

starvation; a ubiquitin protein, a Phytocyanin protein and an AII-domain containing protein. There was a much lower abundance of proteins identified in the IMB211 exudate proteome compared to the R500 exudates proteome. As it appears that the IMB211 line does not secrete as many proteins as the R500 line, this could be indicative of one of the ways that R500 plants have an improved Pi efficiency in low Pi conditions. Due to the small number of proteins identified no further analysis on the IMB211 samples was performed.

4.2.4 PSR in *B. rapa* R500 exudate proteome leads to an increased abundance of phosphatase and RNase proteins

The R500 exudate proteome changed significantly in response to Pi starvation. The 195 proteins identified which significantly changed in abundance were used for further analysis to see what types of proteins change during Pi starvation. TargetP 2.0 was used to determine if there is a SP present on the identified proteins, as it is indicative that the proteins are secreted proteins, the presence of chloroplast, mitochondrial and lumen targeting peptides were also predicted (Fig. 4.4). Of all 593 R500 exudate proteins identified 36% of them showed the presence of a SP, 4% are suspected to be located in the mitochondria, 9% in the chloroplast and 1% in the thylakoid membrane. The 119 proteins more abundant in response to Pi starvation had a higher proportion containing a SP at 45%. While the 76 proteins that were decreased in abundance in response to Pi starvation showed only 14% contained a SP. This increase in proteins with SP identified in response to Pi starvation could indicate that a PSR in *B. rapa* R500 plants leads to an increased secretion of exudate proteins into the surrounding environment.

GO-term enrichment was performed on the proteins that changed in abundance in response to Pi starvation to determine if the expected functions and processes of the exudate proteins changed. Enrichment analysis was performed using PANTHER16.0 using a Fishers exact test with *B. rapa subsp pekinensis* as the background reference and a FDR value < 0.05.

The exudate proteins that were increased in abundance in response to Pi starvation had an enrichment in a variety of biological processes, including a highest FE in glyceraldehyde-3-phosphate biosynthesis and glycerol catabolic process (Fig. 4.5A). There was a variety of molecular functions that were enriched included ribonuclease T(2) activity, peroxidase activity, phosphopyruvate hydratase, GDPD and inorganic diphosphatase activity (Fig. 4.5B).

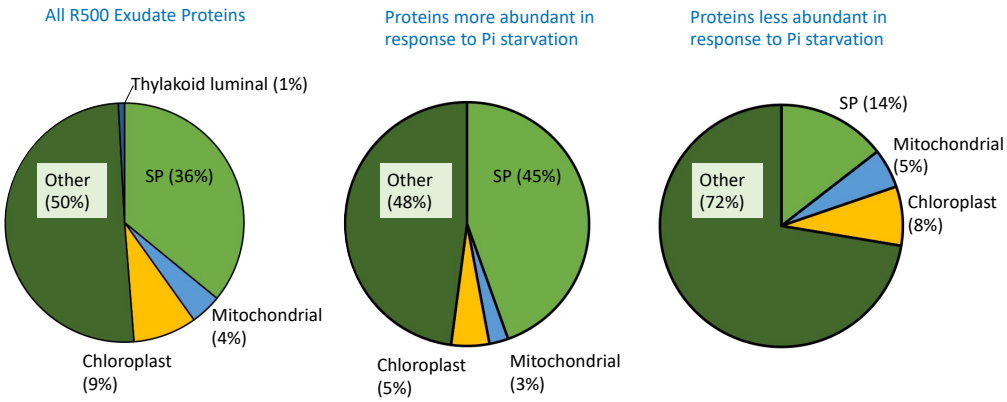

Figure 4.4: R500 exudate proteome predicted localisation: The proteins identified in the R500 exudate samples were analysed using TargetP2.0 to determine predicted protein localisation. All 593 R500 exudate proteins identified predicted localisation represented, the 119 proteins more abundant in response to Pi starvation and the 76 proteins less abundant in response to Pi starvation predicted localisation represented.

The proteins that decrease in abundance in response to Pi starvation had an enrichment in a few biological processes with the highest FE in translational elongation, nucleotide-excision repair and removal of superoxide radicle (Fig. 4.5C). There was an enrichment of a few molecular functions that include elongation factor activity, superoxide dismutase activity and proteosome binding (Fig. 4.5D).

The GO term enrichment analysis indicates that the R500 exudate proteome functions change significantly in response to Pi starvation. These changes in expected activity could be indicative of the plants improved Pi efficiency in low Pi conditions.

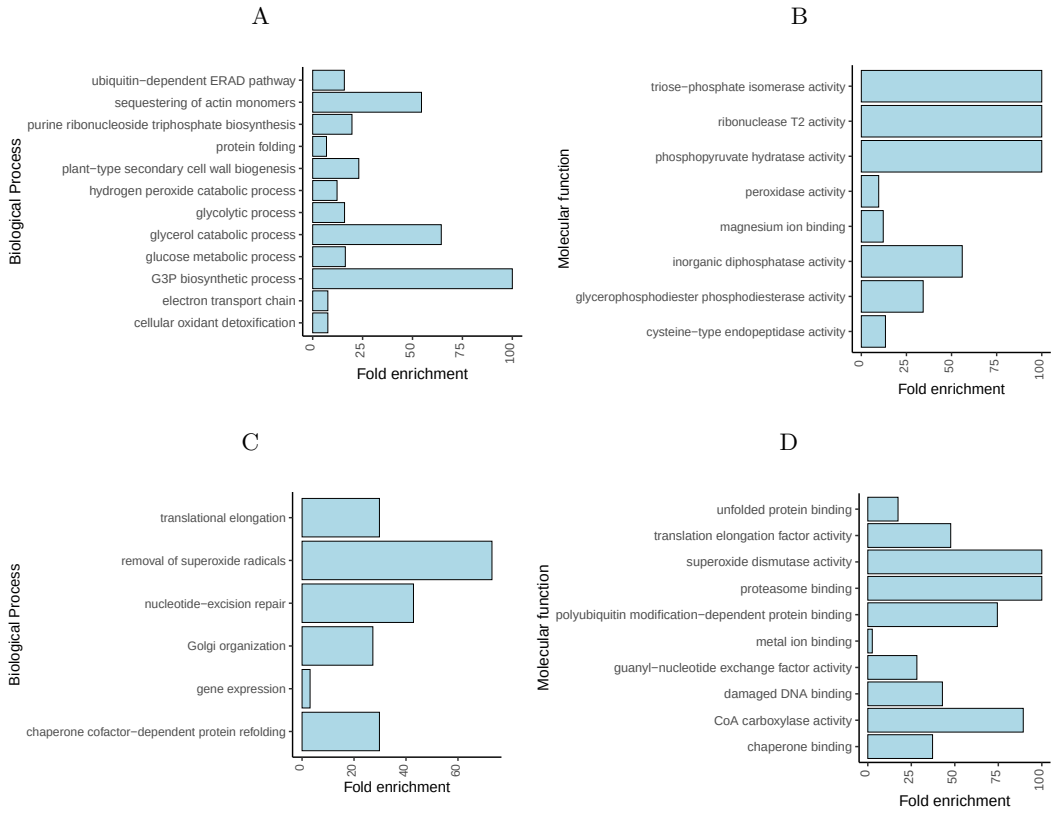

Figure 4.5: *B. rapa* R500 exudate proteome GO-Term enrichment: GO term analysis of the significantly differentially abundant exudate proteins using PANTHER 16.0. Displaying the terms FE which are considered significantly enriched based off a Fishers exact test with FDR > 0.05. A) Biological processes of proteins more abundant in response to Pi starvation. B) Molecular functions of proteins more abundant in response to Pi starvation. C) Biological processes of proteins less abundant in response to Pi starvation. D) Molecular functions of proteins less abundant in response to Pi starvation.

Determining the different types of proteins which were identified in the exudates and whether they changed significantly in response to Pi starvation PANTHER16.0 was used to identify the Panther protein classification of the identified proteins. An enrichment analysis on the protein classes was performed to see if any protein classes are increased during Pi starvation as a PSR to potentially improve the plants Pi efficiency. The exudate proteins which increased in abundance in response to Pi starvation protein classification (Fig.4.6A) showed an enrichment for metabolite interconversion enzymes with these taking up 33% of all PC of the exudates proteins, and hydrolase proteins as a part of metabolite interconversion enzymes was also significantly enriched. There was also an enrichment of cell adhesion molecule proteins.

The exudates proteins that were less abundant in response to Pi starvation protein

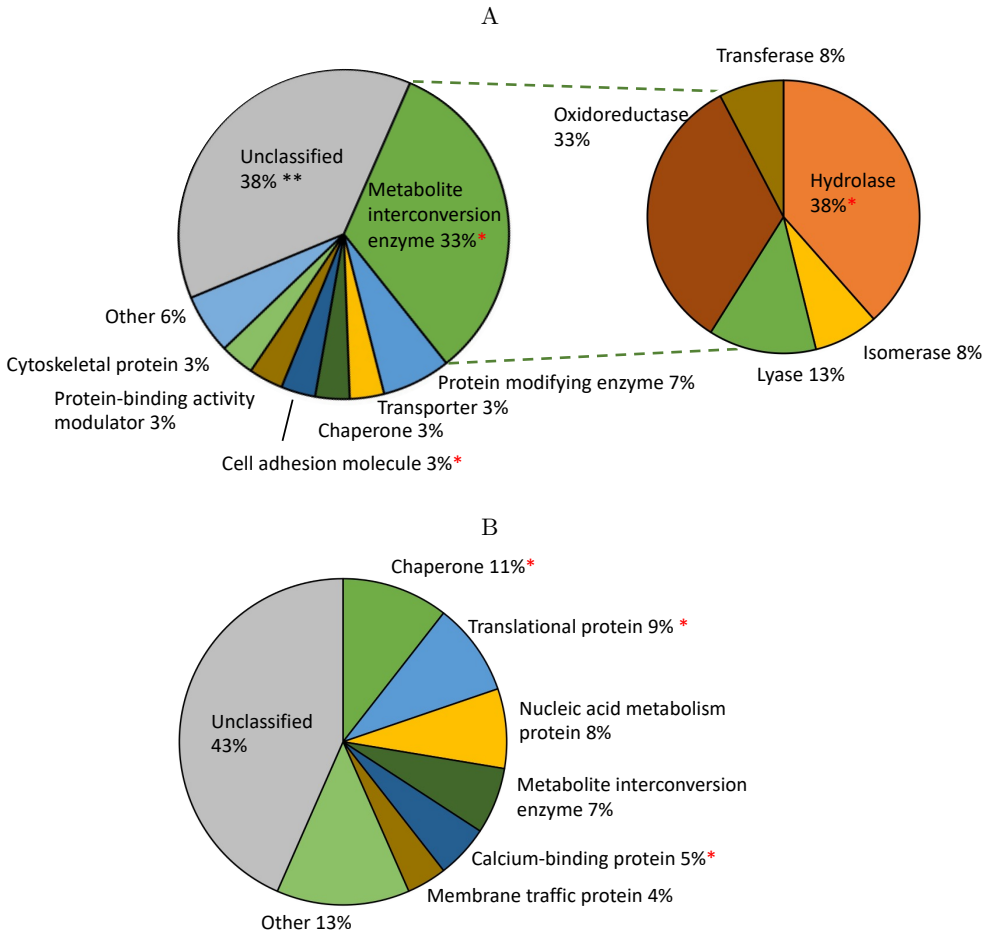

Figure 4.6: Classification of *B. rapa* R500 exudate proteome: The exudate proteins that significantly changed in abundance protein classification were determined using PANTHER16.0 and enrichment analysis of the protein classes was determined using a fisher exact test with FDR < 0.05, * indicate classes which are significantly enriched, ** indicate classes which are significantly under-enriched. A) PC of the exudates proteins increased in abundance in response to Pi starvation. Including the composition of the metabolite interconversion enzymes. B) PC of the exudates proteins decreased in abundance in response to Pi starvation.

classification (Fig. 4.6B) showed that 43% of the proteins identified were unclassified. Protein classes which were significantly enriched were chaperone proteins, translational proteins, and calcium binding proteins. The protein classification of R500 exudate proteins indicates that there is a significant change in the *B. rapa* exudate proteins that respond to Pi starvation.

A subset of the exudate proteins which are significantly changed in abundance in response to Pi starvation are shown in Table 4.1. The proteins and are grouped based on protein group or functions. Five phosphatase proteins are shown to increase in abundance in Pi starved samples, including a PAP (A0A3P5ZT94) which is a homologue to the *Arabidopsis* secretory AtPAP15. Pyrophosphatase proteins increased in

abundance in response to Pi starvation, are also an enriched GO term and protein class. Three RNase proteins were also increased in abundance in response to Pi starvation which matches with the enrichment in ribonuclease T2 activity GO term. There are also an increase in abundance of three GDPD proteins which are involved in lipid metabolic process. Peroxidase proteins are more prevalent in response to Pi starvation while superoxide dismutase proteins are decreased in response to Pi starvation. A few proteins involved in carbohydrate metabolism and proteolysis are also more abundant in the Pi starved exudates.

The changes in the exudate proteins indicates that the exudate proteome of Pi efficient R500 *B. rapa* changes significantly in response to Pi starvation. With a change in abundance of many proteins involved in a variety of functions and processes, some of which are involved in improving plants Pi efficiency as a part of its PSR.

Table 4.1: *B. rapa* R500 exudate proteins significantly changed in response to Pi starvation: A subset of DAP which were identified in the R500 exudate samples. Protein ID and name from uniprot, *Brassica* Chiifu homologue, Log_2 FC LFQ intensity values, Pi starved minus control sample, positive values indicate increased abundance and negative values indicate decreased abundance in response to Pi starvation. TargetP2.0 to determine possible localisation of the proteins. -log P-values from student 2-sample T-test of the FC. * depict uncharacterised proteins so are named based on their closest *Arabidopsis* homologue.

Protein ID	Chiifu homolohue	Protein name	Score	log_2 FC	TargetP	-log P-value
Phosphatase						
A0A3P5ZT94	Bra040363	Purple acid phosphatase 15	19.5	5.5	SP	4.3
A0A3P5YHY8	Bra007016	Inorganic pyrophosphatase 4	5.8	4.7	OTHER	3.7
A0A3P5YVH4	Bra040607	Inositol-phosphate phosphatase	62.8	4.7	OTHER	1.5
A0A3P6CXN1	Bra009396	Inorganic diphosphatase 6	18.4	3.6	cTP	1.8
A0A3P5YNE7	Bra032616	Inorganic diphosphatase 1	2.7	2.6	OTHER	1.9
Lipid metabolism						
A0A3P5ZTK7	Bra026409	GDPD3	32.8	5.7	SP	2.3
A0A3P6CG48	Bra002904	GDPD4	23.1	5.0	SP	2.0
A0A3P6A2W2	Bra019092	GDPD3	7.4	3.8	SP	2.3
Defence and detoxifying						
A0A3P6AH30	Bra019131	Peroxidase	37.9	4.6	SP	1.5
A0A3P5YSV1	Bra032475	Peroxidase	4.6	4.0	SP	2.4
A0A3P5ZY01	Bra001415	Thioredoxin-dependent peroxiredoxin	16.5	3.5	cTP	1.6
A0A3P5ZZ53	Bra000058	Peroxidase	8.2	1.8	SP	1.8
A0A3P6CR30	Bra028761	Peroxidase	36.7	1.4	SP	1.6

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Table 4.1 – *Continued from previous page*

Protein ID	Chiifu homolohue	Protein name	Score	log_2 FC	TargetP	-log P-value
A0A3P6CJA6	Bra034394	Superoxide dismutase [Cu-Zn] 2	175.3	-2.0	cTP	2.3
A0A3P5Y9S3	Bra031642	Superoxide dismutase 1	102.5	-1.7	OTHER	4.3
A0A3P5YUS8	Bra031642	Superoxide dismutase 1	112.8	-1.2	OTHER	3.4
Carbohydrate metabolism						
A0A398AQK8	Bra033549	Glucan endo-1,3-beta-D-glucosidase	62.5	7.3	SP	5.3
A0A3P6A2K2	Bra039757	Glucan endo-1,3-beta-D-glucosidase	16.2	4.2	SP	2.3
A0A3P6C5T5	Bra014636	Glucan endo-1,3-beta-D-glucosidase	32.7	3.6	SP	1.9
A0A3P5Y5U2	Bra007235	Alpha-galactosidase	23.0	3.5	SP	2.0
A0A3P6AXH2	Bra039824	Sinigrinase	14.6	3.5	SP	1.7
A0A3P5ZYS5	Bra000311	Chitin-binding type-1 domain-containing protein	35.5	2.9	SP	2.2
A0A3P5YYA9	Bra034754	Chitin-binding type-1 domain-containing protein	95.4	2.5	SP	1.6
RNase						
A0A3P6AUK7	Bra026570	Ribonuclease T(2) 1	32.7	7.8	SP	5.0
A0A397YEW7	Bra016708	Ribonuclease T(2)	34.2	4.9	SP	4.0
A0A397Z3E6	Bra024795	Ribonuclease T(2) 1	2.5	1.7	SP	1.5
Proteolysis						
A0A3P5YFM9	Bra018067	KDEL-tailed cysteine endopeptidase*	45.4	6.0	SP	2.6
A0A3P5Y4J7	Bra036815	Peptidase A1 domain-containing protein	38.1	5.5	SP	2.9
A0A397Z3N1	Bra025942	Kunitz trypsin inhibitor 5 *	8.6	3.7	SP	2.4
A0A397ZG74	Bra040597	Peptidase A1 domain-containing protein	9.6	3.6	SP	1.8
A0A3P5ZPY7	Bra036815	Peptidase A1 domain-containing protein	50.1	3.6	SP	2.1
A0A3P5YWJ5	Bra022418	cysteine protease RD21C*	41.2	3.5	SP	2.6
A0A3P5ZAB4	Bra033657	cysteine protease RD21B*	12.5	3.5	SP	1.6
A0A397XIF2	Bra015607	SERPIN domain-containing protein	35.9	2.5	mTP	2.8
A0A3P6D322	Bra040749	Cysteine proteinase RD21A*	13.5	2.9	SP	2.5
A0A3P5YB76	Bra036137	Kunitz trypsin inhibitor 6*	104.8	4.0	SP	3.1
A0A3P5ZEV8	Bra011223	Carboxypeptidase	6.6	2.8	SP	1.5
A0A3P5Z2P8	Bra011224	Carboxypeptidase	12.8	2.1	OTHER	1.4

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Table 4.1 – *Continued from previous page*

Protein ID	Chiifu homolohue	Protein name	Score	log_2 FC	TargetP	-log P-value
Misc						
A0A3P5YIS0	Bra038541	PLAT domain-containing protein	4.4	6.0	SP	3.2
A0A3P5ZWG4	Bra022819	Heat shock 70 kDa protein BIP1*	249.8	3.4	SP	2.3
A0A3P6BDF2	Bra001970	Coatomer subunit gamma	7.6	3.5	OTHER	2.5
A0A3P6BGZ9	Bra034627	Coatomer subunit gamma	20.6	2.8	OTHER	1.3
A0A3P5ZQL3	Bra023802	Epidermal patterning factor-like protein	3.0	-3.8	SP	1.3
A0A3P6AIB8	Bra020696	AAI domain-containing protein	3.8	-2.8	SP	2.4
A0A397ZLF8	Bra022364	AAI domain-containing protein	30.2	-2.6	SP	5.1

4.3 Discussion

The Pi efficient *B. rapa* R500 exudate proteome showed a significant change in response to Pi starvation. The exudate proteins identified that increased in abundance during Pi starvation consisted of a higher proportion containing a SP, at 45%, opposed to only 14% the proteins that decreased in abundance containing a SP. This could indicate more secretion of proteins during Pi starvation, as protein transport is largely initiated by the N-terminal signal sequence SP [von Heijne, 1985], while a higher proportion of the proteins detected to be less abundant in response to Pi starvation could have come from damaged or sloughed off cells. The proteins less abundant in the R500 Pi starved samples had a highest increased enrichment GO-term for translation, and CoA carboxylase activity, which indicates that these proteins detected are most likely not secreted proteins.

There were four phosphatase proteins detected to be more abundant in the R500 exudate proteome in response to Pi starvation. One of which was a BrPAP15 protein which is a homologue to the *Arabidopsis* AtPAP15, which is a secreted protein, and has been recorded that when overexpressed in soy bean plants results in an improved Pi efficiency [Wang et al., 2009]. The role of PAP proteins is to release Pi from Po sources and increasing the abundance of these proteins during low Pi conditions is an indicator of the plants improved Pi efficiency to utilise previously unaccessible Pi from Po sources, the P-inefficient IMB211 had no PAPs or any other phosphatase proteins identified in the root exudate proteome.

The inorganic diphosphatase proteins identified to increase in abundance in the R500 exudates, are also called pyrophosphatase proteins in their *Arabidopsis* homologues, did not contain a SP, so it could be that they are not really secreted exudate proteins. The diphosphatase activity molecular function GO term was significantly enriched in response to Pi starvation and they are expected to play a role in Pi recycling and plant adaptation to Pi limiting conditions [Hernández-Domíguez et al., 2012], as well as inositol pyrophosphatase proteins in *Arabidopsis* showing a role in maintaining phosphate homeostasis [Zhu et al., 2019] so a mention of them I think is important, as they are still more abundant in response to Pi starvation.

Another group of proteins that are of interest that increased in abundance in response to Pi starvation in R500 exudates are Ribonuclease proteins. Three Ribonuclease T2 proteins showed an increase in abundance, and there was an enrichment in the ribonuclease T2 activity GO-term. In the previous chapter we determined *Arabidopsis* to have an increased abundance of RNase proteins in their exudate proteome in response to Pi starvation. The degradation of extracellular RNA substrates using RNase proteins to improve Pi availability is a mechanism plants utilise when in low Pi conditions to scavenge for exogenous Pi [Nürnberger et al., 1990]. RNase proteins have been recorded to be secreted to improve the accessibility of Pi by tomatoes to utilise during Pi deficient conditions [Abel et al., 2000]. Nucleic acids are a source of phosphate with approximately 9% of RNA dry weight taken up by phosphate [Raven, 2013]. The extracellular RNase 1 identified previously in tomato cultures by Abel et al. [2000] is a homologue of a *B. rapa* RNase (A0A397Z3E6) protein identified to have increased in abundance in R500 exudate proteome, and is also a homologue of the AtRNase1 (P42818) identified in the previous chapter to have increased in abundance in response to Pi starvation. During a different study by Tran and Plaxton [2008] on the secretome of suspension cells of *Arabidopsis* response to Pi deficient conditions the homologue of the *B.rapa* A0A397Z3E6 RNase was also more abundant in Pi deficient conditions compared to Pi sufficient conditions. This indicates a similar PSR of *Arabidopsis*, the *B.rapa* efficient R500 and tomato plants to secrete RNase in response to low Pi conditions to improve Pi accessibility. The P-inefficient IMB211 did not have any RNase proteins detected within their exudate proteome.

Additionally, in common with the *Arabidopsis* exudate proteome data in the previous chapter there is an increase in the abundance of GDPD proteins in response to Pi starvation in R500 exudate proteome, and an enrichment in GDPD activity GO-term. Increased GDPD abundance could increase the availability of Pi from

phospholipid sources as a part of the lipid remodelling PSR. GDPD catalyses the break down of lipids to release sn-glycerol 3-phosphate (G3P) [Ramaiah et al., 2011]. It has been suggested that GDPD mediated lipid metabolic pathways improve the availability of Pi through its release from phospholipids when in low Pi conditions [Cheng et al., 2011]. Other plant species have also displayed an increased abundance of GDPD proteins as a part of their PSR, with its increased expression leading to improving rice Pi content in low Pi conditions, as well as increased plant biomass when compared to plants which have less GDPD activity [Mehra et al., 2019].

Peptidase proteins were also increased in abundance in response to Pi starvation in R500 exudate samples, with 4 cystine-type peptidase proteins and 3 A1 peptidase domain containing protease proteins increased in abundance. Cystine-type endopeptidase activity was an enriched GO term in response to Pi starvation. Peptidase proteins break down proteins into smaller peptides or amino acids. Peptidase proteins have been shown to be regularly secreted in tobacco plants [Drake et al., 2009, Lallemand et al., 2015], but if they play a specific active role during a plants response to Pi starvation it has not been documented to my knowledge. Plant roots will secrete amino acids, along with other metabolites such as carbohydrates and organic acids in order to provide nutrients and a mutually beneficial environment to the soil microbiome [Canarini et al., 2019, Rolfe et al., 2019], it could be that the plants are providing the amino acids through breaking down rhizobia proteins to encourage the growth of beneficial microbes which in turn could help improve the availability of Pi, but this is just conjecture and further work would be necessary to either confirm or deny this.

Peroxidase proteins were increased in abundance in response to Pi starvation in the R500 exudate proteome, with 4 peroxidase proteins increased in abundance. These proteins are often associated with stress responses and work to reduce peroxide accumulation through substrate oxidation [Lazzarotto et al., 2015]. When suffering nutrient deficiency it is a stressful environment for plants, therefore a stress response of peroxidase is not wholly surprising, but the level of response might indicate a further role of peroxidase within the plant to do more specifically with PSR. In contrast *Arabidopsis* exudate proteome analysed in the previous chapter did not show the same level of peroxidase response, and displayed a decrease in peroxidase abundance in response to Pi starvation. This could be indicative of a different response between the different species to Pi starvation, or it could be the difference in age and growth conditions that lead to the different response, as plants will respond differently to different conditions as they get older. As encouraging as it is

to see similarities in the response of the *B. rapa* plants compared to the *Arabidopsis* plants the species are not wholly comparable therefore can not expect to always see the same responses. Some PSR may be conserved between species but some may be more specific. The IMB211 exudate proteome also contained peroxidase proteins, 10 out of the 79 confidently detected proteins were classed as peroxidases, but there wasn't a difference in abundance in response to Pi starvation, so they could just be a general group of proteins that get secreted when grown hydroponically without a specific role in regards to Pi availability.

4.4 Conclusion

The aims of this chapter were to identify changes in the the P-efficient R500 and the P-inefficient IMB211 *B. rapa* root exudate proteomes in response to Pi starvation. I have determined that R500 roots secrete more proteins than the IMB211 line, and that the R500 root proteome changes significantly in response to Pi starvation, while IMB211 shown little change in their exudate proteome. R500 root exudate proteome changes in a way that could be responsible for improved Pi efficiency. The increased secretion of various proteins such as phosphatases, RNases, and proteins involved in lipid metabolism and proteolysis could improve Pi efficiency by improving Pi availability to the plant. Further work with functional assays is needed to determine more specific roles of the proteins in improving the plants Pi efficiency, as the MS identification of proteins gives abundance and presence, it does not give evidence of activity and whether the presence of these proteins actually improves Pi availability and uptake. However the results do present interesting targets whose functions can be hypothesized and used for further research into their role to improve a plants phosphate efficiency.

Chapter 5

Quantitative Proteomics of Phosphate Starved *B. rapa* Roots

5.1 Introduction

Previous chapters have discussed how the root exudate proteome for both *B. rapa* and *Arabidopsis* change in response to Pi starvation, as well as the *Arabidopsis* phosphoproteomic response to Pi starvation. This chapter covers how the root proteome and phosphoproteome of *B. rapa* R500 changes as a part of its phosphate starvation response (PSR) by looking at the membrane associated proteome, the root soluble proteome, as well as the root phosphoproteome. As it is expected that the root proteome will change in order to improve Pi efficiency, with an increase in abundance of various proteins to improve Pi uptake and utilisation, such as purple acid phosphatases (PAPs) and Pi transporter proteins.

The membrane associated proteins being isolated for separate analysis can provide better insight into the changes of membrane associated proteins in response to Pi starvation. Due to the lower abundance of membrane associated proteins in the whole cell, they can often be missed during MS analysis, which favours higher abundance proteins to be identified, resulting in lower abundance proteins being masked.

The membrane associated proteins include a variety of transporters whose abun-

dance is responsive to Pi levels. For example the the PHT1 plasma membrane phosphate transporters, which are a member of the large major facilitator superfamily (MFS) in *Arabidopsis* have shown an increase in abundance and expression when plants are in low Pi conditions as a part of their PSR to increase Pi uptake, other phosphate transporters that play a role in the distribution of Pi through the rest of the plant to maintain homeostasis, such as the PHO1 transporters are also responsive to Pi levels [Mudge et al., 2002, Nagarajan et al., 2011].

Therefore identifying the changes in membrane associated proteins in response to Pi starvation in *B. rapa*, may provide some insights into what transporters and other membrane associated proteins are responsive to Pi starvation and are therefore involved in improving Pi efficiency. Identifying these Pi responsive targets can be used to further improve plants Pi efficiency. *Arabidopsis* studies have utilised overexpression phosphate transporter lines which have resulted in improved Pi uptake in Pi deficient conditions by utilising high affinity AtPHT1 transporters in tobacco cultures [Mitsukawa et al., 1997]. Rice also has overexpression lines of the OsPHT1;3 transporter which results in improved Pi uptake [Chang et al., 2019].

Phosphoproteomic analysis of the *B. rapa* roots could allow the identification of potential signalling players specific to root tissues. In contrast to the phosphoproteomic analysis performed on *Arabidopsis* which looked at whole seedlings. Pi signalling in plants involves root to shoot signalling and the different tissues respond to Pi starvation in different ways, shoots having improved Pi utilisation efficiency, while roots induce an improved Pi uptake efficiency, therefore the signalling events that occur in response to Pi starvation are likely to be different in different tissues. I analysed the phosphoproteome of *B. rapa* roots in response to Pi starvation to determine if there was a change in phosphopeptides to identify potential signalling targets.

5.1.1 Aims

The aims of this chapter were to determine how the proteome of R500 *B. rapa* roots change in response to Pi starvation and to identify the proteins which change in abundance, as these changes could indicate proteins which are involved in improving *B. rapa* phosphate efficiency. To achieve this aim I induced a PSR in *B. rapa* R500 plants to harvest the root material for the following analysis;

- Quantitative analysis of the *B. rapa* root membrane associated proteome in response to Pi starvation.

- Quantitative analysis of the *B. rapa* root soluble proteome in response to Pi starvation.
- Quantitative analysis of the *B. rapa* whole root phosphoproteome in response to Pi starvation.

5.2 Results

In order to analyse proteomic changes in *B. rapa* roots in response to Pi starvation I needed to establish a PSR. *B. rapa* R500 was grown hydroponically until 31 days old before they were harvested. Half of the plants had been starved of Pi for 11 days to initiate a PSR while the other half were maintained in Pi sufficient conditions. The root material was harvested and the proteins extracted, 1 mg of total extract protein was allocated for phosphopeptide enrichment, the rest was used to isolate the membrane associated proteins to separate from the remaining root soluble proteins. The membrane associated proteome, soluble proteome and phosphoproteome root samples were analysed using MS (Fig. 5.1).

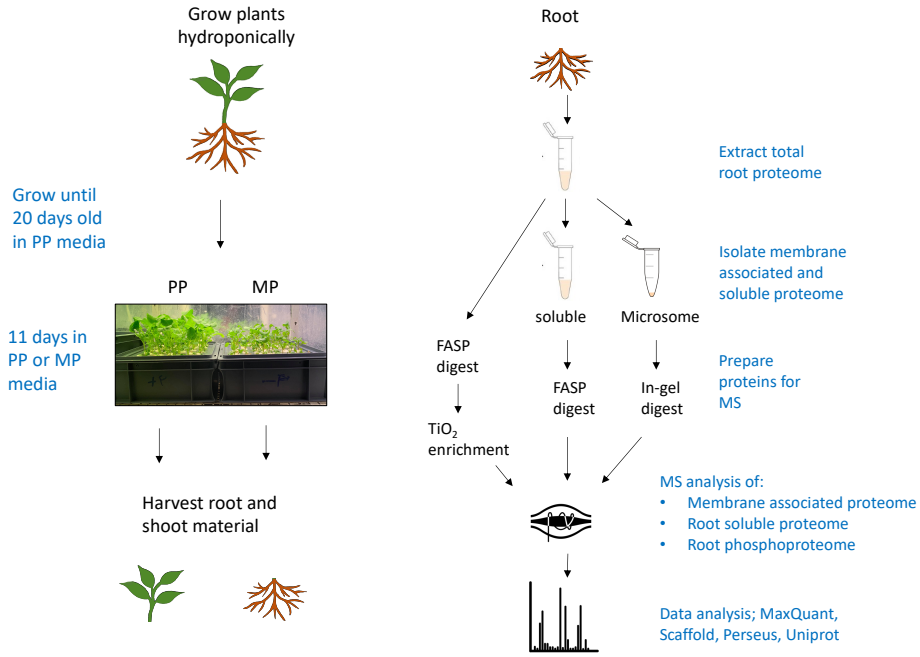

Figure 5.1: Preparation of the *B. rapa* root proteome: *B. rapa* R500 line were grown hydroponically in Pi sufficient (PP) media until 20 days old, after which half were transferred into Pi deficient (MP) media while the other half remained in PP media for a further 11 days to initiate a PSR. The root material was harvested and root proteins were extracted and prepared for MS and the data analysed using a variety of software and web tools.

5.2.1 The APase activity in *B. rapa* root proteins increases in response to Pi starvation

In plants APase activity increases as a part of a PSR, as seen in *Arabidopsis* in chapter 1 and by Wang et al. [2014a]. I therefore wanted to see if the membrane associated and the soluble protein extracts from *B. rapa* roots displayed an increase in APase activity in response to Pi starvation. To test this I performed an in gel APase activity assay, where the protein extracts are separated on a gel and then incubated in APase activity buffer containing BNAP and Fast Black K, where APase activity is indicated by red coloration. The assay gels show that both the membrane associated and the root soluble protein extracts have a greater APase activity in the Pi starved samples, indicated by the darker red coloration (Fig. 5.2). The membrane associated protein extracts showed coloration in high molecular weight at approximately 130 KDa in the Pi starved samples while there was no coloration in the control samples (Fig. 5.2A). The soluble protein extracts showed coloration at high molecular weight of approximately between 130 KDa and 170KDa with no coloration at these weights in the control samples, there also was coloration at 70 KDa in the Pi starved samples while there was very little coloration in the control samples at this weight, there was also slight coloration at a low molecular weight of approximately 35 KDa in both the control and the Pi starved samples (Fig. 5.2B).

These results show that Pi starvation lead to an increase in APase activity in *B. rapa* roots. I conclude that the Pi deficient conditions induced a PSR response in the roots of *B. rapa* plants, these root proteins were then used for further analysis using MS.

5.2.2 PSR in *B. rapa* leads to a significant change in the membrane associated root proteome with an increased abundance of phosphate transporters and phosphatase proteins

The proteins in the roots of plants are expected to change in response to Pi starvation to improve the plants Pi efficiency. I want to identify how the membrane associated proteome of *B. rapa* roots changes in response to Pi starvation and identify what proteins change in abundance which could be attributed to improving the plants Pi efficiency. To analyse how the proteins change *B. rapa* plants were grown hydroponically and a PSR was induced. The membrane associated proteins were isolated from root protein extracts to be identified using MS for quantitative analysis, with four replicates.

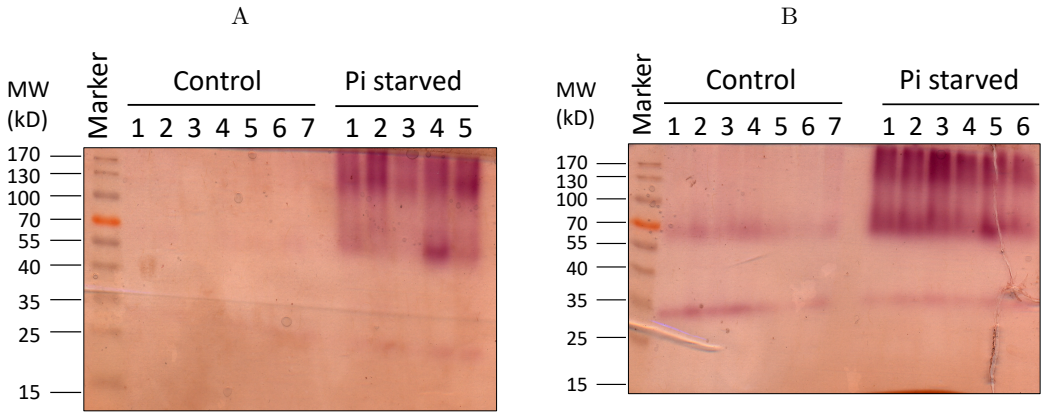

Figure 5.2: *B. rapa* root proteins APase activity in response to Pi starvation: In gel APase activity assay on protein extracts from *B. rapa* R500 roots grown in Pi deficient or control conditions. A) Membrane associated root proteins. B) Soluble root proteins. 2.5 ug of protein extract is separated on native PAGE-gel then incubated in APase activity buffer containing BNAP and Fast Black K, where APase activity is indicated by red coloration.

After the MS analysis a total of 191,642 MS spectra were recorded and assigned to 3,766 proteins. The proteins were filtered by the removal of low confidence proteins, anomalous proteins, potential contaminants, reverse values, and further filtered so that the protein had to be present in at least three of the biological replicates to be kept for analysis. This left 2,242 proteins identified in the root membrane associated proteome for further analysis.

Using the LFQ intensity values the identified proteins were further analysed to determine any changes in the membrane associated proteome in response to Pi starvation. A PCA was performed to determine how different the samples were (Fig. 5.3A). The PCA shows that there is a clear separation of the Pi starved samples compared to the control samples, with a component 1 value of 46.9%, indicating there is a difference in the membrane associated proteome of *B. rapa* roots in response to Pi starvation.

A student two-sample t-test was performed on the LFQ intensities and used to determine the significantly differentially abundant proteins in response to Pi starvation, using an FDR cut of value of 0.05 and s0 value of 0.1. A volcano plot was made to represent this data, plotting $\log_2$ FC difference, with Pi starved sample values minus control sample values, against -log p-value, points in red show the significantly DAP (Fig. 5.3B). Of the 2,242 proteins identified 454 proteins were considered significantly differentially abundant. 311 proteins were more abundant in response to Pi starvation and 143 proteins were less abundant. The proteins

determined to be differentially abundant were selected and the $\log_2$ LFQ intensity levels were normalised using Z-score then used to generate a heat map (Fig. 5.3C). The samples clustered clearly based on their Pi condition with a distinction between the proteins that were more or less abundant in response to Pi starvation. These results show that the membrane associated proteome in the roots of *B. rapa* plants changes significantly as a part of their PSR.

A subset of proteins that are differentially abundant are represented in Table 5.1, due to the high number of proteins that changed in abundance only proteins groups

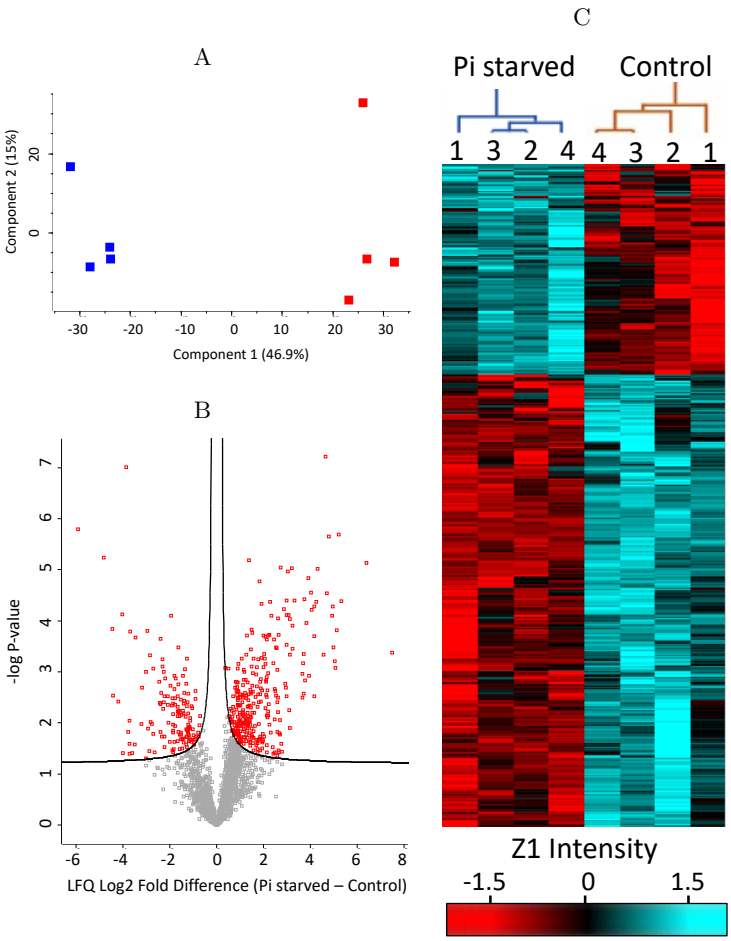

Figure 5.3: Quantitative analysis of *B. rapa* membrane associated root proteome in response to Pi starvation: The proteins identified in the membrane associated proteome were analysed in Perseus using LFQ intensity values. A) PCA plot of membrane associated protein samples. Pi starved samples are highlighted in red, control samples are highlighted in blue. B) Volcano plot of membrane associated proteins, using a student two sample t-test to identify proteins significantly changed in abundance, $\log_2$ fold change is plotted against -log P-value. Red points show the 454 proteins which are significantly changed in abundance in response to Pi starvation, FDR:0.05 s0:0.1. C) Heat map of the 454 significantly changed proteins. $\log_2$ LFQ intensity values normalised using Z-score for graphical representation.

of interest to phosphate stress with a $\log_2$ FC higher than 1 are displayed to keep the table at a manageable level, predicted number of transmembrane domains of the proteins are also represented as these are a common membrane association. In response to Pi starvation there was an increase in abundance of multiple phosphatase proteins, including four PAP proteins, there was also an increase in a number of transporters including six phosphate transporters and ATPase proteins. Of the proteins that decreased in abundance in response to Pi starvation 83 out of 141 of them were ribosomal proteins (58.9%) while none of the proteins more abundant in response to Pi starvation are considered ribosomal proteins, these proteins are not represented in the Table 5.1.

Table 5.1: *B. rapa* membrane associated root proteins that significantly change in response to Pi starvation: A subset of membrane associated root proteins that change in abundance in response to Pi starvation grouped under protein family's or processes. Protein ID's and names taken from uniprot. $\log_2$ FC of LFQ intensity values, Pi starved samples - control samples, positive values indicate increase abundance negative values indicate decreased abundance. TMMHM2.0 was used to predict TM domains. * Indicate uncharacterised proteins so are named based of the closest *Arabidopsis* homologue

Protein ID	Chifu homologue	Protein name	Score	$\log_2$ FC	TM
Phosphatase					
A0A3P5YTG9	Bra026890	Purple acid phosphatase 1	48.3	6.4	1
A0A397XZY9	Bra006951	Purple acid phosphatase 22	20.3	5	0
A0A3P5Y9A6	Bra037302	Purple acid phosphatase 10	158.5	4.3	1
A0A397YG61	Bra030553	HAD superfamily acid phosphatase	8.6	3.9	0
A0A3P5YVH4	Bra040607	Inositol-phosphate phosphatase	9.2	3.2	0
A0A3P5YHY8	Bra007016	Inorganic diphosphatase	46.7	3.1	0
A0A3P6BCC8	Bra014262	Sucrose-phosphatase	7.7	3	0
A0A3P5YNG5	Bra036797	Phosphoinositide phosphatase SAC7	9.2	2.7	0
A0A3P5ZT94	Bra040363	Purple acid phosphatase 15	10.6	2.2	1
A0A397Z4M8	Bra033713	vegetative storage protein-like	54.8	1.9	0
A0A397Y478	Bra016653	H(+)-exporting diphosphatase	312.3	1.15	14
Transporters					
A0A3P6BLF7	Bra003719	Phosphate transporter 1;9	56.1	5.3	12
A0A3P6B1W6	Bra035978	Phosphate transporter 1;3	6.9	5.2	11
A0A3P5XY90	Bra035978	Phosphate transporter 1;3	102.2	4.9	11
A0A3P5YS68	Bra005070	Phosphate transporter 1;4	60.7	4.2	11
A0A397ZBE3	Bra033676	Phosphate transporter 1;3	22.5	3.2	11
A0A3P6AN32	Bra035977	Phosphate transporter 1;3	323.3	3.1	11
A0A3P5ZHH7	Bra038242	sugar transporter 4	5.7	2.6	12
A0A3P5YFW4	Bra018150	glycerol-3-phosphate transporter 1*	4	2.6	12
A0A3P6AVN6	Bra012176	Sugar transporter ERD6-like 7*	13.7	2.5	12
A0A3P5YET4	Bra025848	tonoplast monosaccharide transporter1	97	2.1	10
A0A3P6ADM6	Bra022511	High-affinity nitrate transporter	31.5	1.9	1
A0A3P5ZP12	Bra011604	tonoplast monosaccharide transporter2	34.3	1.6	11
A0A397ZRW7	Bra040987	V-SNARE domain-containing protein	22.7	1.5	1
A0A397ZW09	Bra022969	Plug_translocon domain protein	24.4	-0.8	10
A0A3P5YQU6	Bra007603	PIP1B	46.9	-1.1	5
A0A397YK69	Bra015028	Ammonium transporter	11	-1.2	9

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Table 5.1 – *Continued from previous page*

Protein ID	Chifu homologue	Protein name	Score	log_2 FC	TM
A0A397Y9X9	Bra026991	oligopeptide transport	9.7	-2.2	11
A0A3P5ZXK4	Bra023103	AquaporinPIP2-2	7.5	-3.8	6
Phosphoenolpyruvate carboxylase					
A0A3P5ZNL1	Bra001575	PEPC	75.5	7.5	0
A0A3P5YR48	Bra004710	PEPC	31.1	3.7	0
A0A397Y6V5	Bra030945	PEPC	323.3	2.3	0
A0A3P5Z150	Bra024410	PEPCK	5.5	1.8	0
A0A3P5Z0Z4	Bra011792	PEPCK	28.5	0.9	0
ATPase					
A0A3P5Y6P1	Bra007524	Plasma membrane ATPase	19.2	4.2	8
A0A3P6A909	Bra024100	Plasma membrane ATPase	72.7	2.9	10
A0A3P5Z0H7	Bra018116	ABC transporter A	9.47	2.8	6
A0A3P6BUN0	Bra035163	Plasma membrane ATPase	10.7	2.5	8
A0A3P6A8L4	Bra024100	Plasma membrane ATPase	105.2	2.3	8
A0A3P6C2R7	Bra030503	ABC transporter B	79.2	1.61	9
A0A3P5Y5N0	Bra007319	Calcium-transporting ATPase	179	1.4	8
A0A397YT31	Bra035190	ADP,ATP carrier protein	27.1	1	11
A0A3P6BJ09	Bra010712	V-type proton ATPase subunit a	160.1	0.9	6
A0A3P5Y981	Bra026951	V-type proton ATPase subunit C	153.5	0.9	0
A0A3P6AIJ8	Bra017632	V-type proton ATPase subunit H	23.7	0.9	0
A0A3P5ZHS4	Bra011504	V-type proton ATPase subunit H	53.9	0.9	0
Defence and detoxifying					
A0A3P6AEC4	Bra019132	peroxidase 44	76.1	3.3	1
A0A3P6AH30	Bra019131	Peroxidase 44	189	3	1
A0A3P5ZL62	Bra013943	peroxidase 44	185.7	3	1
A0A3P6A8D4	Bra023639	peroxidase 57	133.1	2.5	1
A0A397YN05	Bra030607	Peroxidase 2	56.7	2.3	0
A0A3P5XW82	Bra037849	peroxidase 73	82.7	2.3	0
A0A3P5ZEM6	Bra006423	Peroxidase 57	150.9	2	1
A0A3P6CJ07	Bra030606	Peroxidase 1	18	1.9	0
A0A3P6CY46	Bra008715	Peroxidase 56	79.4	1.8	1
A0A3P6BKE5	Bra016127	peroxidase 12	232.4	1.8	1
A0A3P5YDT6	Bra039816	peroxidase 32	83.1	1.7	0
A0A398AI65	Bra011771	Peroxidase 50	31.5	1.5	0
A0A3P5ZZ53	Bra000058	Peroxidase 22	6	1.5	0
A0A3P5Y9S3	Bra031642	Superoxide dismutase	74.2	1.4	0
A0A3P6CR30	Bra028761	peroxidase 52	57.4	1.3	0
A0A3P5ZNZ5	Bra011170	Peroxidase 45	18.7	1.2	0
A0A397ZGC8	Bra039137	Peroxidase 27	189	1.3	1
A0A3P5Z164	Bra011683	peroxidase 49	14.3	1.2	0
A0A397XYT9	Bra037877	Peroxidase 38	191.6	1	0
A0A398A6K4	Bra023509	Peroxidase 56	68.5	1	1
A0A3P5ZFZ3	Bra020782	Glutaredoxin-dependent peroxiredoxin	18.7	1	0
A0A3P5YUS8	Bra031642	Superoxide dismutase	79.8	0.9	0
A0A3P5Z8H2	Bra013863	Superoxide dismutase	15.7	-2.	0

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Table 5.1 – *Continued from previous page*

Protein ID	Chifu homologue	Protein name	Score	$\log_2$ FC	TM
Misc					
A0A3P6AA35	Bra017798	Patatin	5.3	5.1	0
A0A398AN20	Bra031878	PLAT domain-containing protein	15.7	5	1
A0A397Z166	Bra009763	Probable 6-phosphogluconolactonase	21.7	5	0
A0A3P6BJY4	Bra003273	Glucan endo-1,3-beta-D-glucosidase	78.7	5	1
A0A3P6AUK7	Bra026570	Ribonuclease T(2) 1	11.8	3.8	1
A0A3P5ZKC4	Bra006090	Sucrose-phosphate synthase	7.7	3.7	0
A0A397XKW7	Bra002289	Sucrose-phosphate synthase	4.7	3	0
A0A397YEW7	Bra016708	Ribonuclease T(2)	23.3	2.6	0
A0A3P5XVJ2	Bra024437	NADH dehydrogenase 4	7.3	1.5	0
A0A3P5Z4Y5	Bra013717	Reticulon-like protein	21	-1	3
A0A397L2N5	Bra019285	Reticulon-like protein	4.9	-2.3	3
lipid remodelling					
A0A3P6A2W2	Bra019092	GDPD3	14	2.7	0
A0A3P6C0P5	Bra015928	GDPD5	25.8	2.6	1
A0A3P5ZTK7	Bra026409	GDPD3	46.4	0.5	0
A0A397ZQK6	Bra016862	Phospholipase A1	8.9	1.6	0

The proteins that changed in abundance were used for further analysis to identify what types of proteins change in response to Pi starvation. The functions of the proteins that significantly change are suspected to play a role in improving the plants Pi efficiency, with membrane proteins likely having a role in improving Pi uptake efficiency. GO term and protein class enrichment analysis was used to get an idea of what functions or processes we see a significant enrichment in during Pi starvation. PANTHER16.0 was used for the enrichment analysis with *B. rapa sbsp pekineasis* as a background reference using a Fisher exact test with a FDR correction of < 0.05. Proteins which increased in abundance in response to Pi starvation had a variety of biological processes significantly enriched including phosphate ion transport, regulation of intracellular pH, sulfolipid biosynthetic process, sucrose biosynthetic process as some of the processes with the highest fold enrichment (Fig. 5.4A). A variety of molecular functions were also enriched in the proteins increased in abundance in response to Pi starvation that could be involved with improved Pi efficiency, including ribonuclease activity, Pi transmembrane transporter activity, acid phosphatase activity and PEPC activity, as well as ATPase activity, cellular compartments also enriched were involved in ATPase complex (Fig. 5.4B,C). While GO terms related to regulation of transcription and DNA binding are under enriched. Non membrane bound organelle protein GO terms were also underenriched.

The proteins that decreased in abundance in response to Pi starvation had fewer

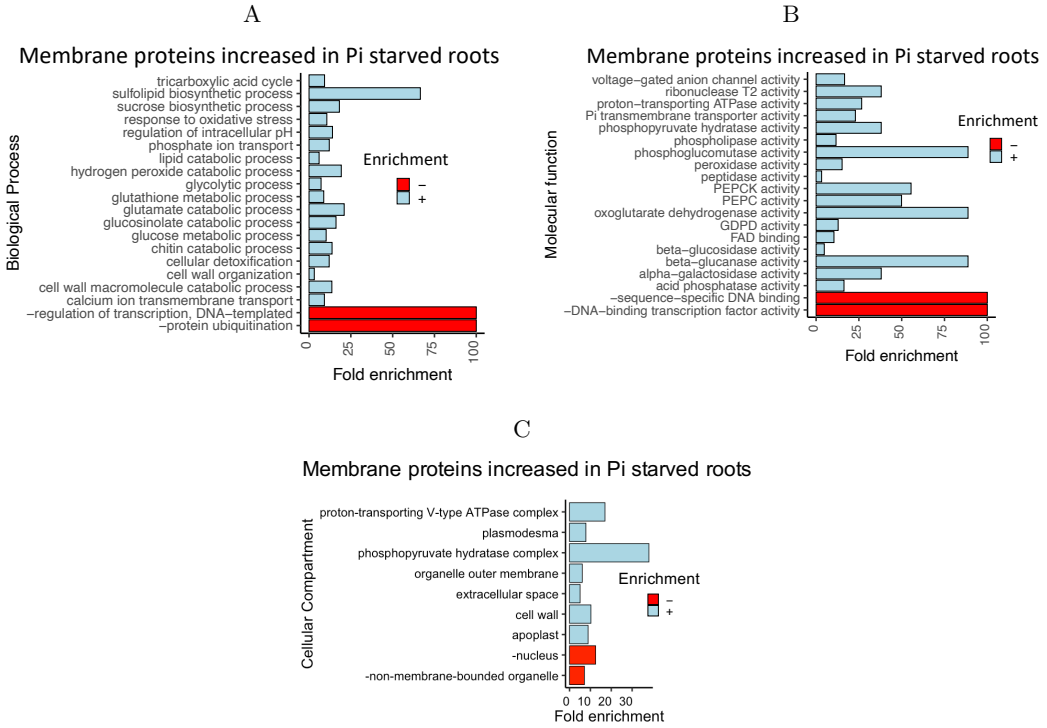

Figure 5.4: *B. rapa* membrane associated root proteome GO-Term enrichment: GO term analysis of the membrane associated root proteins that increased in abundance during Pi starvation using PANTHER 16.0. Displaying the terms FE which are significantly enriched based on a Fisher exact text with FDR < 0.05, bars coloured in blue denote terms that were positively over-enriched, bars coloured in red denote terms which are negatively under-enriched. A) BP GO term analysis. B) MF GO terms. C) Cellular compartment GO terms.

GO terms enriched, the biological processes that were enriched include ribosome assembly, cytoplasmic translation and maturation of SSU-rRNA. The molecular functions that were enriched in the proteins decreased in abundance are structural constituent of ribosome, RNA and ribosomal binding, which correlates with 58.9% of the proteins being ribosomal proteins.

The GO term enrichments in proteins changed in response to Pi starvation indicate a significant change in the predicted functions of the proteins that are increased in abundance in response to Pi starvation. These could indicate a role in the membrane associated root proteins for improving *B. rapa* Pi efficiency.

To classify the different types of proteins which changed in abundance in response to Pi starvation, protein class designated by PANTHER was used with enrichment analysis to determine if there was an enrichment of certain classes of proteins which could indicate what proteins may play a role to improve Pi efficiency. Enrichment analysis was performed using PANTHER 16.0 with a Fishers exact test with FDR correction of FDR < 0.05. The membrane associated proteins that increased in abundance in response to Pi starvation classification revealed that 203 of the 311 proteins had a PC designation, leaving 33% of the proteins unclassified (Fig. 5.5A). The PC that were significantly enriched were transporter proteins, and metabolite interconversion enzymes which consist of multiple enzyme types that were also enriched including hydrolase proteins which make up 35.9% of the metabolite interconversion enzymes classified, isomerase, lyase and oxidoreductase enzymes were also enriched. The proteins which decreased in abundance in response to Pi starvation were classified, (Fig. 5.5B). 113 of the 143 proteins has a PC designation, the only PC that was significantly enriched was the translational proteins class, which makes up 57 % of the proteins.

The membrane associated proteins of *B. rapa* roots change significantly in response to Pi starvation. With a change in abundance of many proteins that are involved in a variety of functions and processes which could be involved in improving the plants Pi efficiency by improving the Pi uptake efficiency as a part of the *B. rapa* PSR.

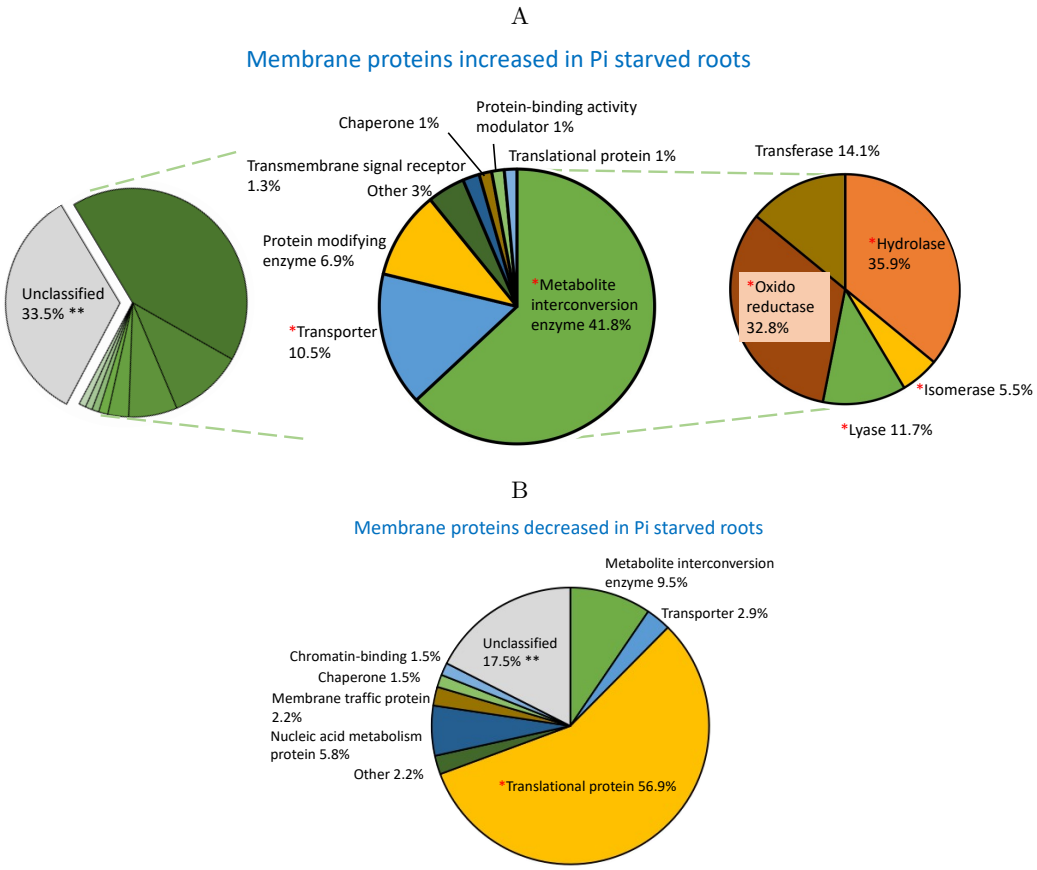

Figure 5.5: Classification of *B. rapa* membrane associated root proteome: The membrane associated proteins that significantly changed in abundance protein classification were determined using PANTHER16.0, enrichment analysis of the protein classes was performed using a Fisher exact test with FDR < 0.05, * indicate classes which are significantly enriched, ** indicate classes which are significantly underenriched. A) Proteins which increased in abundance during Pi starvation, 33.5% were unclassified so showing the proportion of the 66.5% of the proteins protein classes which did have a Panther protein class designation, and the proportion of the different metabolite interconversion enzymes are also shown. B) Proteins that decreased in abundance during Pi starvation protein classes.

5.2.3 PSR in *B. rapa* causes to a significant change in the soluble root proteome composition

The membrane associated proteins of *B. rapa* roots change significantly in response to Pi starvation. I expect that the rest of the root proteome will also change to further adapt to the Pi deficient conditions. To test this hypothesis a PSR was induced in *B. rapa* plants and the root proteins were extracted, after the membrane associated proteins were isolated the rest of the root soluble proteins were identified using MS for quantitative analysis.

After the MS analysis 266,086 MS spectra were recorded and assigned to 2,988 proteins. The proteins were filtered for potential contaminants, reverse values, and

low confidence proteins and further filtered so that the proteins had to present in at least three of the replicates of one growth conditions to be kept for analysis. This left 1,361 proteins identified in the root proteome.

Using the LFQ intensity values the identified proteins were further analysed to determine any changes in the root proteome. A PCA was performed (Fig. 5.6A) and shows that there is a good clustering of the samples within the same conditions but that there is a distinct separation of the Pi starved root proteins compared with the control samples, with a component 1 value of 46.2%. Indicating a difference in the *B. rapa* soluble root proteome in response to Pi starvation.

A student two-sample t-test was performed on the proteins LFQ intensity values to determine the proteins that significantly change in abundance in response to Pi starvation using an FDR cut off value of 0.05 and s0 value of 0.1. A volcano plot was made to represent this data, plotting $\log_2$ fold difference against -log P-value, points in red show the significantly differentially abundant proteins (Fig. 5.6B). Of the 1,361 proteins identified 542 proteins were considered significantly changed in abundance, 186 proteins are increased in abundance in response to Pi starvation while 356 proteins decreased in abundance. The proteins determined to be differentially abundant were selected and the $\log_2$ LFQ intensity levels were normalised using Z-score then used to generate a heat map (Fig. 5.6C). The samples clustered based Pi conditions and there is a distinction between the proteins which are more or less abundant. These results show that there is a significant difference in the root proteome of *B. rapa* plants in response to Pi starvation.

I wanted to see how the predicted functions of the root proteins which changed in response to Pi starvation changed. These changes could be indicative of how *B. rapa* respond to Pi starvation to improve their phosphate efficiency, by increasing the abundance of certain proteins that function in such a way as to better adapt to insufficient Pi availability.

GO-Term enrichment analysis was performed using PANTHER16.0 with a Fisher exact test with a FDR value <0.05 to determine terms that are significantly represented. In the proteins that increased in abundance in response to Pi starvation, there was a variety of biological processes enriched (Fig. 5.7A), terms with the highest FE included UTP biosynthetic process, protein glutathionylation and ketone catabolic process. A variety of molecular functions were enriched in response to Pi starvation including ribonuclease T2 activity, acid phosphatase activity, PEPC activity, dipeptidyl-peptidase activity and lactoylglutathione lyase activity having

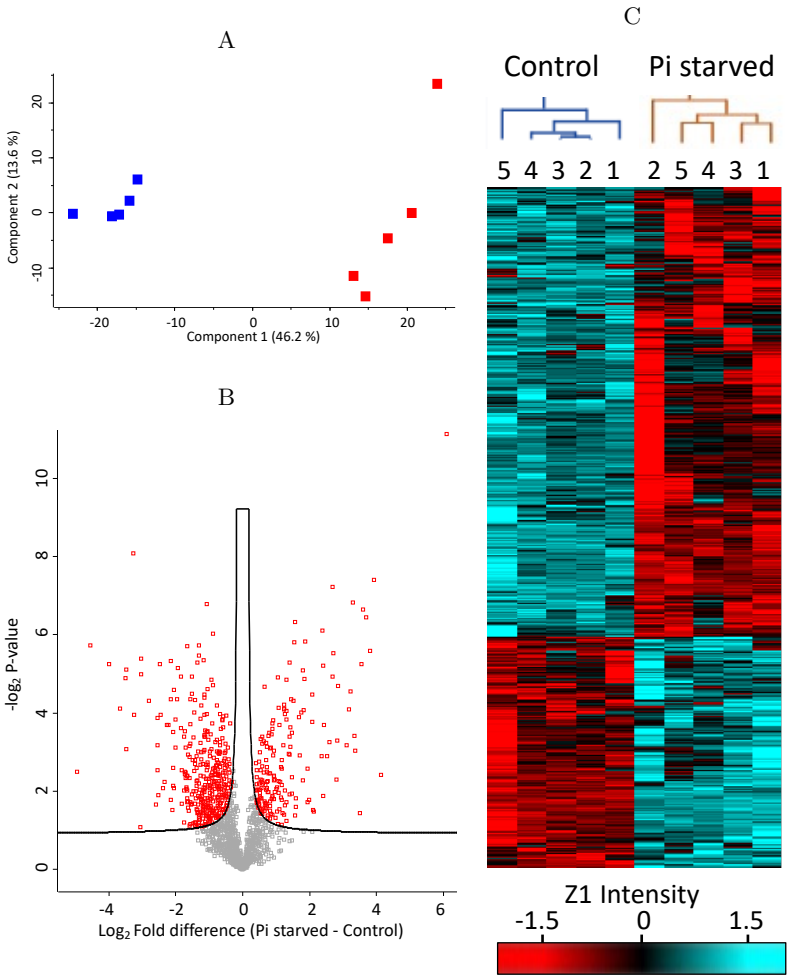

Figure 5.6: Quantitative analysis of *B. rapa* soluble root proteome in response to Pi starvation: The proteins identified in the root soluble samples were analysed in Perseus using LFQ intensity values. A) PCA plot of root proteome, Pi starved samples highlighted in red, and control samples are highlighted in blue. B) Volcano plot of root proteins, using a student two sample t-test to identify proteins significantly changed in abundance between conditions, $\log_2$ fold change is plotted against -log P-value. Red points show the 542 proteins which are significantly changed in abundance in response to Pi starvation. FDR: 0.05 s0: 0.1. C) Heat map for the 542 significantly changed proteins during Pi starvation. log*2* LFQ intensity values normalised using the Z-score for graphical representation.

the highest FE (Fig. 5.7B).

The proteins which decreased in abundance in response to Pi starvation had a variety of biological processes enriched, the most enriched including succinyl-CoA metabolic process and ribosomal subunit assembly (Fig. 5.7C). There was an enrichment in numerous molecular functions, including phosphogluconate 2 dehydrogenase activity and acyl-CoA dehydrogenase activity which the highest FE (Fig. 5.7D). GO-term enrichment analysis indicates a significant enrichment of the functions of *B. rapa* root proteins which change in response to Pi starvation, this could be attributed to

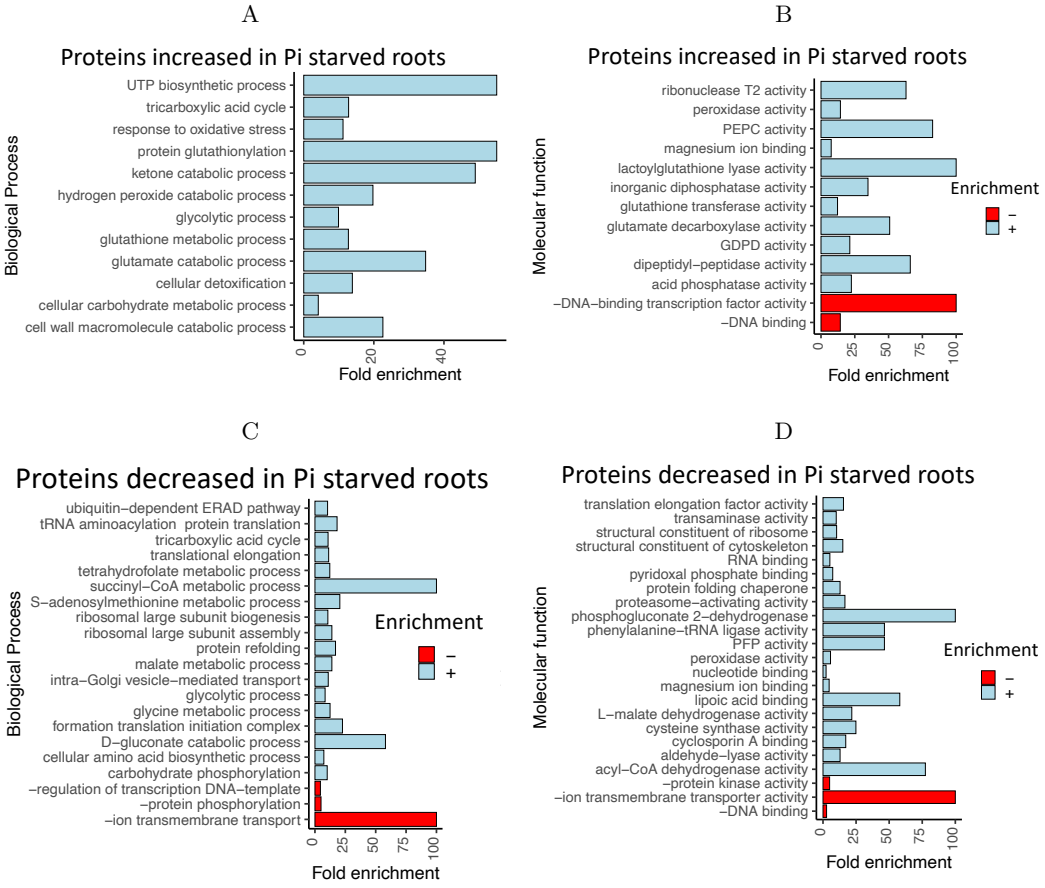

Figure 5.7: *B. rapa* soluble root proteome GO-term enrichment: GO term analysis of the significantly differentially abundant root proteins Using PANTHER 16.0. Displaying the terms FE which are considered significantly enriched based off a Fisher exact text with FDR < 0.05. A, B) GO term analysis of proteins increased in abundance in response to Pi starvation. C, D) GO term analysis of proteins decreased in abundance response to Pi starvation. A, C) Biological process GO-term enrichment. B, D) Molecular functions GO-term enrichment. Bars in blue denote terms that were positively enriched. Bars in red denote terms which are negatively under-enriched.

the plants trying to improve their Pi efficiency as a part of its PSR.

The Panther protein classification of the proteins which change in abundance in response to Pi starvation are expected to change to adapt to the insufficient Pi availability. Protein classification was determined using PANTHER16.0 and enrichment analysis was performed using a Fishers exact test. The proteins that increased in abundance in response to Pi starvation showed that 62 out of the 186 proteins (33%) did not have a panther protein classification, the majority of the proteins identified were metabolite interconversion enzymes at 47%, of these 34.5% were hydrolase proteins, both of which were significantly enriched protein classes, lyase and oxidoreductase proteins were also enriched (Fig. 5.8A).

Of the 356 proteins that decreased in abundance in response to Pi starvation 18% did not have a Panther protein class designation. The most common protein classes were metabolite interconversion enzymes and translational proteins, both of which were significantly enriched, chaperone proteins were also enriched in the proteins that decreased in response to Pi starvation. Of the metabolite interconversion enzymes; ligase, lyase, oxidoreductase and transferase enzymes were also significantly enriched (Fig. 5.8B).

The protein classification and Go-term enrichment analysis indicate that the types of proteins within the *B. rapa* soluble root proteome change significantly in response to Pi starvation. The changes likely being a part of a PSR which could help to improve the plants Pi efficiency, diverting energy and resources away from some processes in favour of others to adapt to low Pi availability.

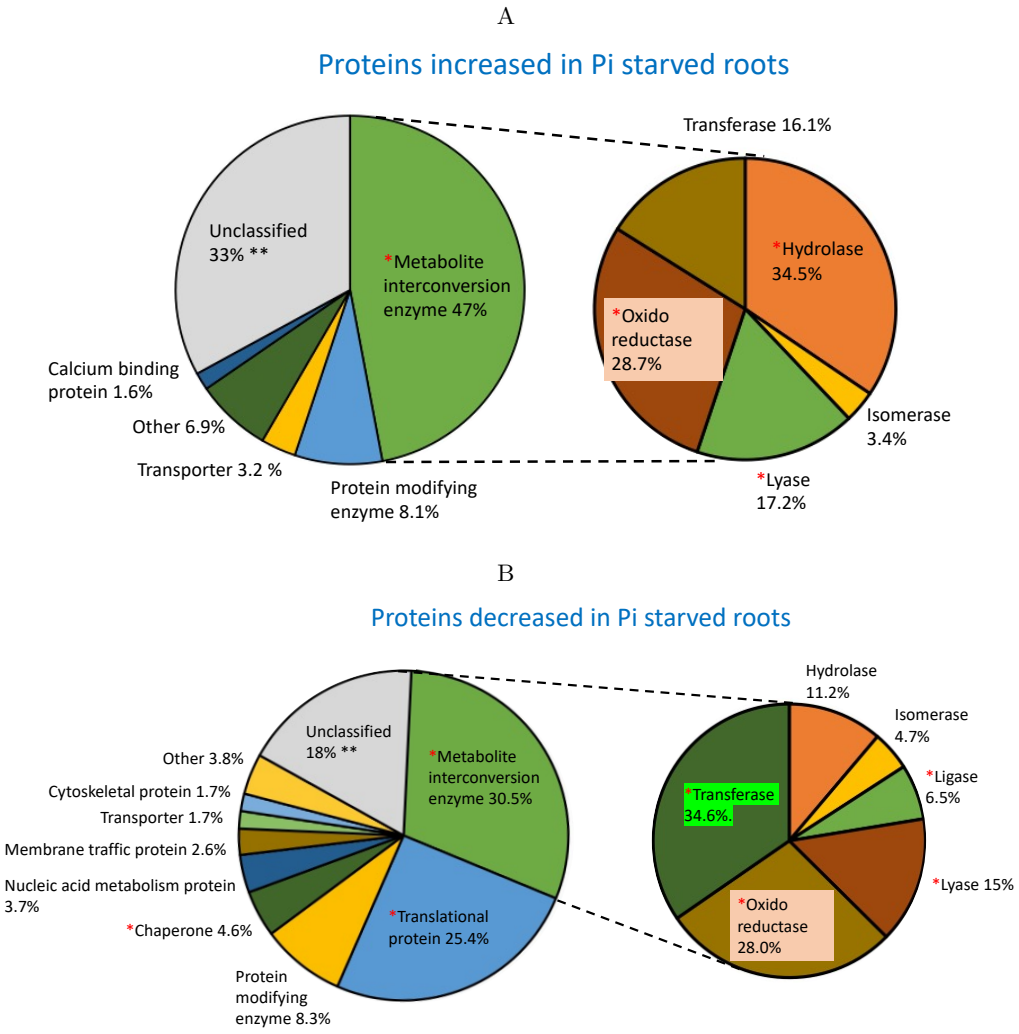

Figure 5.8: Classification of *B. rapa* soluble root proteome: The root proteins that changed in abundance in response to Pi starvation protein classes were determined using PANTHER 16.0 and enrichment analysis was performed using a Fisher exact test with FDR correction value < 0.05. A) PC of the proteins that increased in abundance in response to Pi starvation. B) PC of the proteins that decreased in abundance in response to Pi starvation. * Denotes PC which are significantly over enrichment. ** Denotes PC which are significantly under-enriched.

5.2.4 PSR in *B. rapa* roots results in an altered phosphoproteome indicating a change in signalling events

The root proteome changes significantly in response to Pi starvation, and we also expect that signalling events will change in response to Pi availability. Phosphorylated proteins are often involved in signalling pathways and can be identified during phosphoproteomic analysis by enriching for phosphopeptides. Phosphopeptide enrichment was performed using TiO_2 on total root protein extracts from *B. rapa*

R500 plants grown hydroponically after a PSR was induced, the phosphopeptides were identified using MS for quantitative analysis.

After the MS analysis a total of 61,300 spectra were recorded which were assigned to 985 different phosphopeptides with a phospho enrichment efficiency of 44% . These peptides were filtered by the removal of low confidence peptides, potential contaminates, and a localisation score of less than 0.75, and further filtered so that the phosphopeptide had to be present in all three biological replicates of a growth condition. This left 382 phosphopeptides identified which are assigned to 303 different proteins.

Using the LFQ intensity values these phosphopeptides were further analysed to determine changes in the phosphoproteome in response to Pi starvation. A PCA was performed (Fig. 5.9A) and shows a clear distinction between the phosphoproteome samples from Pi starved plants compared to control samples with a component 1 value of 75%, while the sample replicates grouped together. Indicating that the phosphoproteome of *B. rapa* roots are altered in response to Pi starvation.

A standard two-sample t-test was performed on the phosphopeptides $\log_2$ LFQ intensities to determine significantly differentially abundant phosphopeptides in response to Pi starvation, using a FDR value of 0.05 and s0 value of 0.1. A volcano plot was used to represent this data, plotting the $\log_2$ FC difference of Pi starved intensities minus the control LFQ intensities plotted against the -log p-value (Fig. 5.9B) points highlighted in red represent the phosphopeptides significantly changed in abundance. Of the 382 phosphopeptides detected 246 which significantly changed in abundance in response to Pi starvation. 6 phosphopeptides were identified to increase in abundance in response to Pi starvation while 240 decreased in abundance in response to Pi starvation. The phosphopeptides determined to be significantly changed were selected and the $\log_2$ LFQ intensity values normalised using z-score to create a heat map (Fig. 5.9C). The samples in the different conditions grouped together and there is a clear distinction between the peptides that increased or decreased in abundance. These results show that there is a significant change in the phosphoproteome of *B. rapa* roots in response to Pi starvation, with 64% of the identified phosphopeptides being significantly changed in abundance. Indicating a change in signalling events involved during a PSR in *B. rapa*.

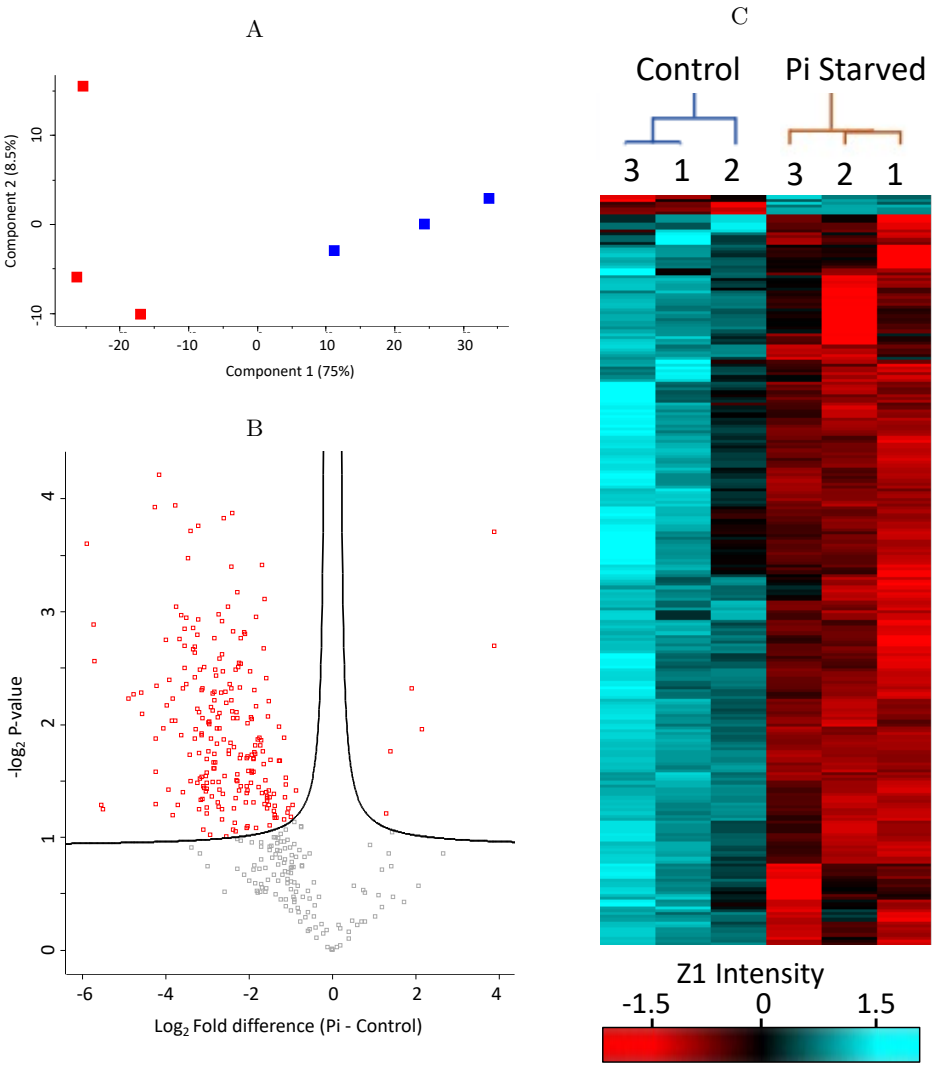

Figure 5.9: Quantitative analysis of *B. rapa* phosphoproteome in response to Pi starvation: The phosphopeptides identified in the phosphoenriched *B. rapa* root samples were analysed in Perseus using LFQ intensity values. A) PCA plot of phosphopeptides, Pi starved samples indicated in red, control samples indicated in blue. B) Volcano plot of phosphopeptides, using a student two sample t-test to identify phosphopeptides significantly changed in abundance, $\log_2$ fold change is plotted against -log P-value. Red points show the 246 phosphopeptides which are significantly changed in abundance in response to Pi starvation, FDR:0.05 s0:0.1. C) Heat map for the 246 significantly changed phosphopeptides, $\log_2$ LFQ intensity values normalised using the Z-score for graphical representation.

The majority of the phospho modifications identified in the root phosphoproteome are on serine residues at 91%, 3% are phosphotyrosine, and 6% are phosphothreonine (Fig. 5.10A). The majority of the proteins detected are mono phosphorylated, 93%, 5% have two phospho modifications and 2% have three phospho modifications (Fig. 5.10B). These values do not change significantly in the phosphopeptides that change in abundance in response to Pi starvation.

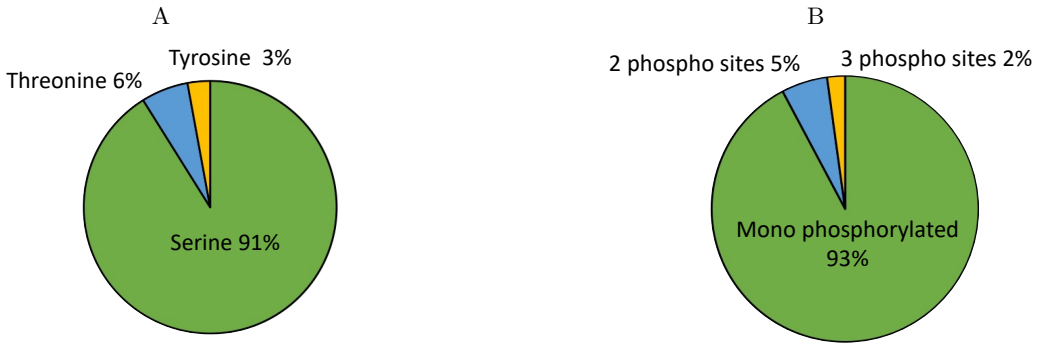

Figure 5.10: *B. rapa* root phosphomodification summary: Of all 382 phosphopeptides identified in root phosphoproteome. A) Percentage of phosphosites identified assigned to serine, threonine and tyrosine. B) Percentage of phosphpeptides detected with either 1, 2 or 3 phosphorylations.

The changes in phosphorylated proteins and peptides could help to indicate what proteins are involved in signalling events during Pi starvation. The phosphopeptides which increased in abundance in response to Pi starvation are shown in Table 5.2. There were only six phosphopeptides identified that increased, two of which were PEPC proteins, a transmembrane transporter monosaccharide sensing protein 1, Foie-gras-1 and chromo domain containing proteins.

In contrast there was a large amount of phosphopeptides identified to have decreased in abundance in response to Pi starvation. 240 phosphopeptides decreased in abundance assigned to 200 different proteins, a subset of the phosphopeptides identified which have at least a $\log_2$ FC of 1.5 are displayed in Table 5.2 to make the table more manageable. The proteins that decreased in abundance underwent GO term enrichment analysis using PANTHER16.0 to determine if there were certain types of proteins that had a decrease in phosphopeptide abundance, a Fishers exact test with FDR cut off value of 0.05 was used to determine any significant enrichments. Biological processes enriched involved vesical transport and vesicle targeting as well as sucrose catabolic process, endocytosis and mRNA processing. Some of the proteins involved in these processes are included in Table 5.2. Protein classes that were also enriched were GTPase-activating proteins, RNA splicing factor and non-receptor serine/threonine protein kinase proteins, the identified phosphopeptides related to some of these proteins are also depicted within Table 5.2. To my knowledge none of the phosphopeptides detected that changed significantly in response to Pi starvation have been documented from previous studies (according to uniprot as of november 2021).

Table 5.2: *B. rapa* root phosphopeptides that changed in abundance in response to Pi starvation: A subset of the phosphopeptides identified in *B. rapa* root phosphoproteome are shown, and grouped based on if they increase or decrease in abundance in response to Pi starvation, and by biological processes and protein classification. log_2 fold change of the peptides and -log P value of the calculated FC. Protein ID's and names from uniprot. The *Brassica* chifu homologue. Site is the position within the protein phosphorylated. * Indicates proteins that were uncharacterised so are named using their closest *Arabidopsis* homologue.

Protein ID	Chifu homologue	Protein name	Sequence window	Log_2 FC	-Log p-value	Site
Increased in abundance during Pi starvation						
A0A3P5Z9C2	Bra027293	PEPC 3	EKMA**S**IDAQ	3.9	2.7	S11
A0A3P5YR48	Bra004710	PEPC 2	KKMA**S**IDAQ	3.9	3.7	S11
A0A3P6AN63	Bra023629	Chromo domain-containing protein	AGPY**S**QLKK	2.1	2.0	S147
A0A3P6CVE3	Bra002991	Foie-gras_1 domain-containing protein	DSPV**S**PKQD	1.9	2.3	S904
A0A3P5YET4	Bra025848	Monosaccharide-sensing protein 1	QQQS**S**LGLR	1.4	1.8	S276
A0A3P6BFM4	Bra038997	Uncharacterised protein	ARRT**S**LVPIV	1.3	1.2	S297
Decreased in abundance during Pi starvation						
GTPase Proteins						
A0A3P5ZI83	Bra040376	Ras-related protein RABA5b *	KAQL**S**VNRV	-4.2	2.3	S192
A0A3P5ZXN0	Bra000005	Arf-GAP domain-containing protein	AKSK**S**SEDI	-3.9	1.4	S186
			GFGS**S**PGPA	-2.8	2.1	S235
A0A3P5YP41	Bra024999	Arf-GAP domain-containing protein	AKSI**S**SAQF	-3.2	1.8	S308
A0A3P5Z306	Bra013265	Arf-GAP domain-containing protein	TNAK**S**ISSA	-2.9	2.2	S297
A0A3P6BZE9	Bra032698	Arf-GAP domain-containing protein	QRSM**S**APSL	-2.6	1.4	S407
			SGSR**S**PPYE	-2.4	3.9	S150
A0A3P5ZVR2	Bra035433	Dynamin GTPase	RQSF**S**EGSL	-2.1	2.8	S743
Serine-threonin kinase						
A0A397ZEJ3	Bra029850	3-phosphoinositide-dependent protein kinase 2 *	SQSA**S**PERD	-3.0	1.7	S335
A0A3P5Y6T9	Bra007738	Protein kinase domain containing protein	VGSR**S**AGGG	-2.4	1.8	S27
A0A3P6AL91	Bra031892	Protein kinase domain containing protein	SGNL**T**NRVI	-2.3	1.5	T198
A0A3P5Z071	Bra039146	SNF1-related protein kinase KIN10 *	ESVA**S**PVSN	-1.1	1.5	S363
A0A3P5Z163	Bra011605	Calcium-dependent protein kinase 5 *	SLNI**S**MRDA	-1.1	1.2	S541

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Table 5.2 – *Continued from previous page*

Protein ID	Chifu homologue	Protein name	Sequence window	Log_2 FC	-log p-value	Site
A0A3P6BYC2	Bra010744	Serine/threonine-protein kinase STY46 *	EKKR**S**PTGG	-3.8	2.2	561
A0A3P5Z856	Bra013762	Protein kinase domain-containing protein	ERSE**S**EHQA	-1.6	1.5	501
A0A3P6D926	Bra008742	Protein kinase domain-containing protein	ESAL**S**PERA	-1.8	1.8	S373
			SADL**S**PKGS	-1.5	1.1	S548
A0A3P5ZWA0	Bra028333	Protein kinase domain-containing protein	EIVT**S**PGRD	-3.0	1.5	S94
A0A3P6ADZ7	Bra013139	Protein kinase domain-containing protein	ARPA**S**PLHN	-1.9	2.0	S131
A0A397XQM0	Bra009420	Calcium-dependent protein kinase 1*	TQPE**S**PKPE	-1.5	2.1	S87
RNA-splicing factor, mRNA processing						
A0A3P5YYP5	Bra026037	RRM domain-containing protein	KPVL**S**PRRR	-5.7	2.6	S220
			RLPDSPPRR	-2.9	1.0	S240
A0A397ZHP8	Bra014741	RRM domain-containing protein	RY**S**PPYY**S**P	-4.3	3.9	S6
				-4.2	4.2	S11
A0A3P5YGN2	Bra024624	Serine/arginine-rich splicing factor RSZ21*	PLRR**Y**SRSP	-3.8	3.0	Y154
A0A3P6AY30	Bra008295	SKIP_SNW domain-containing protein	KASG**S**PPVP	-3.4	3.7	S202
			PVMH**S**PPR	-3.4	3.7	S210
A0A3P5Z4U3	Bra013895	Serine/arginine-rich splicing factor RS40	RRR**S**P**S**PY	-2.9	1.9	S233
				-2.9	1.9	S235
			RERT**S**PDYG	-3.2	1.3	S262
			GRGG**S**PVAA	-2.9	2.1	S251
			RERR**S**PDDS	-2.6	2.3	S311
A0A3P5ZXZ0	Bra029146	Serine/arginine-rich splicing factor RS41*	EKEA**S**PENG	-2.5	2.4	S325
A0A3P6ACN5	Bra012646	RRM domain-containing protein	KLSE**S**PEPK	-2.4	2.3	S279
Transport/vesicle localisation						
A0A3P5YPH5	Bra004750	ENTH domain-containing protein	QRKF**S**EQNI	-3.8	3.9	S286
A0A3P5YJZ1	Bra017483	Protein transport protein sec16	QRFP**S**LDNI	-3.5	2.7	S1304

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Table 5.2 – *Continued from previous page*

Protein ID	Chifu homologue	Protein name	Sequence window	Log_2 FC	-log p-value	Site
A0A3P5XY46	Bra027093	Secretory carrier-associated membrane protein 3	NSRL**S**PLPP	-3.2	1.5	S34
A0A3P6CIG1	Bra034252	ENTH domain-containing protein	KRSR**S**YGDM	-3.2	2.1	S217
A0A3P5Z2E2	Bra011333	ENTH domain-containing protein	KRSR**S**FGDV	-1.6	1.4	S195
A0A397XVN6	Bra013969	Peroxisome biogenesis protein 19-2*	LGSL**S**SKQL	-2.7	1.2	S98
A0A3P5ZEL2	Bra039641	Myosin-3*	EANL**S**PERE	-2.0	1.5	S1123
Sucrose catabolic Process						
A0A3P6BDN3	Bra036761	Alkaline/neutral invertase CINV1	GLHD**S**PRGR	-2.9	1.4	S61
A0A397ZES2	Bra034413	Alkaline/neutral invertase CINV1	SRHD**S**PRGR	-1.8	3.0	S54
A0A397ZZL8	Bra000681	Alkaline/neutral invertase CINV2	DMAH**S**PGSR	-1.5	1.4	S61

Phosphosites contain common motifs which can be informative about what kinase proteins could be responsible for the phosporylation, as well as indicate phosphatase proteins involved in dephosphorylation, therefore can indicate what proteins are involved in signalling pathways. Motif analysis using MoMo motif-x algorithm can show what motifs are enriched in a data set, using a Fisher exact test with a P-value threshold of $< 1e^{-}6$ and *B. rapa* as a background reference [Cheng et al., 2019]. The Motif analysis on all deteced phosphopeptides within the root phosphoproteome showed that there were five common motifs that occured. Of these motifs none were enriched in the phosphopeptides more abundant in the Pi starved roots. The phosphopeptides that decreased in abundance in response to Pi starvation had four motifs that were enriched (-SP-, R–S, P-SP, -TP-), displayed in Table 5.3.

The *B.rapa* root phosphoproteome is significantly changed in response to Pi starvation. Indicating Pi related signalling events mediated by phosphorylation changes drastically in response to Pi availability, these changes could be a part of the plants PSR to improve Pi efficiency to adapt to low Pi availability.

Table 5.3: Motif analysis of *B. rapa* root phosphoproteome: Phosphopeptides detected in *B. rapa* root phosphoproteome analysed using MoMo motif-x to determine enriched motifs, with a Fishers exact test, P-value threshold of <10e-6, using *B. rapa* background reference. Arrows indicate the motifs that are enriched in the phosphopeptides that significantly decreased in response to Pi starvation.

Motif	Foreground matches	Background matches	Score	Significantly changed phosphopeptides
......SP.....	169	77678	72.9	↓
...R..S.....	83	78461	24.3	↓
....P.SP.....	49	6272	128.3	↓
...K..S.....	37	80323	6.2	
......TP.....	14	43176	12.07	↓

5.3 Discussion

The proteome of *B. rapa* roots changes significantly in response to Pi starvation, some of these proteins which change in abundance could be attributed to improving the plants Pi efficiency. Roots improve their tolerence to low Pi availabilty by improving their uptake efficiency. The membrane assocoiated root proteins were isolated for analysis to identify what membrane proteins change in response to Pi starvation, as they could be responsible for improving Pi uptake efficiency. Other plant species, such as *Arabidopsis* and rice, increase the induction of phosphate transporters (PHT1 family) in response to Pi starvation as a part of their improved uptake efficiency [Fang et al., 2009, Remy et al., 2012]. These transporters allow for the increased uptake of available Pi from the environment as well as the remobilization of Pi within cells, such as stored Pi within vacuoles.

The *B. rapa* membrane associated proteome has an increased abundance of transporter proteins in response to Pi starvation, with transporter activity being a significantly enriched PC, as well as phosphate ion transport process and Pi transmembrane transporter activity GO terms being significantly enriched, contributed to by the 6 BrPHT transporters that are significantly increased in abundance (Table 5.1). 4 BrPHT1;3, BrPHT1;9 and BrPHT1;4 proteins were increased in abundance, which are predicted to be localised to the plasma membrane based off the localisation of their *Arabidopsis* homologues. These proteins and have previously been reported to be responsive to Pi deprivation in *Arabidopsis* roots resulting in increased expression [Hazel et al., 2014]. AtPHT1;9 is more expressed in the endodermis and xylem and are involved in root to shoot translocatin of phosphate [Hazel et al., 2014]. AtPHT1;3 is reported to be located within the epidermis near the main root tip and predominantly in the trichoblasts epidermal cells of lateral roots [Mudge

et al., 2002], and root hairs have already been reported to be important in the role of nutrient uptake [Gahoonia and Nielsen, 1998]. AtPHT1;4 is expressed within the epidermis, cortex, stele, and root tips [Misson et al., 2004, Mlodzinska and Zboinska, 2016, Mudge et al., 2002], suggesteding overlapping functions of the Pi transporters AtPHT1;3/4, which are high affinity plasma membrane transporteres to play a role in the uptake of Pi from the envinroment. *Brassica napus* PHT1;4 protein, which is a homologue to the *B. rapa* PHT1;4 (A0A3P5YS68) with 99.2% sequence identity indentified to increase in abundance in response to Pi starvation, has also been reported to promote Pi uptake during Pi deprivation [Ren et al., 2014]. The Rice OsPHT1;3 also improves phosphate uptake efficiency during low Pi conditions in rice [Chang et al., 2019]. Hence, increasing the expression and abundance of Pi transporters within plant roots during low Pi conditions contributes to improve the Pi uptake efficiency of the plant and is a PSR that appears to be conserved amongst multiple plant species.

ATPase proteins were shown to increase in abundance in response to Pi starvation in the membrane associated proteome. Proton transporting ATPase activity, and regulation of intracellular pH are significantly enriched and can be attributed to the increase in ATPase proteins. ATPase membrane proteins function to pump protons out of the root cells into the surrounding area, this change in gradient drives PHT1 transporters, which act as proton coupled symporters to then import Pi and protons into the root cells [Ullrich-Eberius et al., 1981]. *Arabidopsis* plants have shown an increased H^+ flux activity during low Pi conditions attributed to an increased expression of plasma membrane ATPase proteins AtAHA1,2 and 7 [Yuan et al., 2017]. Two homologues of the AtAHA2 protein are increased in abundance in the *B. rapa* membrane associated root proteome (A0A3P6A909, A0A3P6A8L4), and one AtAHA2 homologue (A0A3P5Y6P1) is also increased in abundance, indicating a similar response of the ATPase proteins during Pi starvation in both *B. rapa* and *Arabidopsis*.

There was also an increased abundance of the sn-glycerol 3-phosphate (G3P) transporter (A0A3P5YFW4) in response to Pi starvation, which may correlate with the GDPD proteins identified to increase in abundance in response to Pi starvation. Three GPDP proteins were significantly more abundant *B. rapa* root proteome and root exudate proteome. These proteins catalyse the break down of phospholipids to release G3P. Seeing the transporter increased in abundance as well as seeing an increased abundance of the proteins which catalyze the substrates availability is a very nice correlation. The *Arabidopsis* G3P transporter homologue (Q9C5L3) has also

previously been documented to be induced in response to Pi starvation [Ramaiah et al., 2011].

There were some membrane associated proteins that were only seen in the membrane associated proteome, like the phosphate transporter proteins (PHT) and the ATPase proteins, which had high number of transmembrane domains, which indicates some success in the seperation of membrane associated proteins from the rest of the root soluble proteins. However there were also a number of proteins that were common to the proteins identified within the root soluble proteome, as well as identified in the root phosphoproteome (Fig. 5.11A). There were 35 proteins identified in all three proteomes. There was quite a large commonality in the proteins identified in both the membrane associated proteome and root soluble proteome (1,011 protein), indicating either multiple localisations for some proteins, or more likely a not perfect isolation of all membrane associated proteins, and some membrane associated proteins might only be weakly associated therefore, remain in the root soluble proteome during isolation. Of the proteins that significantly changed in abundance between the root proteomes in response to Pi starvation, there were some that increased or decreased in abundance in both the membrane associated proteome and soluble proteome, but there were also some cases where the proteins that increased in one proteome decreased in the other in response to Pi starvation (Fig. 5.11B). These changes could indicate a change in the location of the proteins as a response to Pi starvation.

One protein increased in the root soluble proteome while decreasing in the root membrane associated proteome, an uncharacterised USP domain containing proteins (A0A3P5ZB72) whose function is unknown. Four proteins increased in abundance in the membrane associated proteome while decreasing in abundance in the root soluble proteome, two of which were sinigrinase proteins (A0A3P6ABK4, A0A3P5ZBI9). Sinigrinase proteins catalyse the breakdown of glucosinolates to glucose, a sinigrinase protein (A0A3P6AXH2) in the *B. rapa* R500 exudate proteome was also identified to increase in abundance in response to Pi starvation. The release of sugars could be involved in a PSR, carbohydrate metabolic process is an enriched GO term process in both the membrane associated and root soluble proteins which increased in response to Pi starvation.

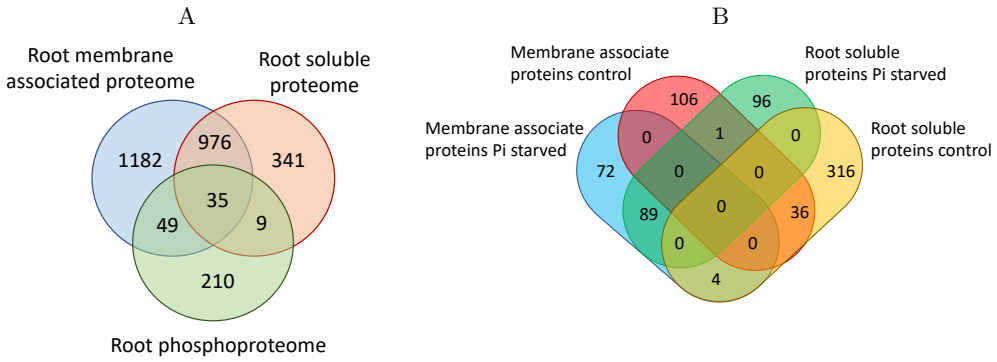

Figure 5.11: Comparison of the *B. rapa* root proteomes and phosphoproteome: A) The proteins in common identified in the root membrane associated proteome, soluble proteome and phosphoproteome. B) Proteins identified that changed in abundance in response to Pi starvation in the root membrane associated proteome and root soluble proteome.

PAP proteins significantly increase in abundance in response to Pi starvation in both the root membrane associated and soluble proteomes. There was also a clear increase in the activity of the root proteins in response to Pi starvation not just an increase in abundance, shown by in-gel APase activity assays on the protein extracts (Fig. 5.2). PAP1, 10, and 15 are identified in both the soluble and membrane associated proteomes, all have a transmembrane domain predicting that the majority of the c-terminal of the protein, which includes the active sites is to be located extracellularly, as predicted by TMMHM2.0. These PAPs are suspected to be root membrane assiciated PAPs that function to improve Pi availability to be taken up into cells. BrPAP22 is only identified to increase in abundance in the membrane associted proteome, while BrPAP26 increases in abundance only in the soluble root proteome. The *Arabidopsis* homologue AtPAP26 is documented to be a vacuolar phosphatase protein [Veljanovski et al., 2006]

Ribonuclease T2 proteins 1 and 3 (A0A3P6AUK7, A0A3P6AUK7) increase in abundance in both the membrane associated proteome and membrane soluble proteome in response to Pi starvation. Ribonuclease proteins function catalyse the breakdown of RNA, and to remobalise phosphate especially during low Pi conditions [Bariola et al., 1994].

Sucrose is a vital player in plant development, widley distributed in plants it plays a role in signalling. Two enzymes vital in the role of sucrose synthesis are sucrose phosphate synthase (SPS) and sucrose synthase proteins [Huber and Huber, 1996]. They function to generate sucrose-6-phospahte (S6P) from fructose-6-phosphate, as part of sucrose biosynthetic process. Two BrSPS proteins are increased in abun-

dance in response to Pi starvation in the membrane associated proteome. A Sucrose phosphatase protein was also identified to increase in abundance in response to Pi starvation, which functions to remove S6P, it is suspected that SPS and sucrose phosphatase work together as a complex [Huber and Huber, 1996].

The number of phosphopeptides identified in the root phosphoproteome that decrease in abundance in response to Pi starvation is much higher than the number that increases. A potential theory for this is the use of the P used in the phosphorylation of proteins instead being remobilised for nutrient use and a change in phosphate utalisation efficiency. However considering the relatively low amount of P that gets used for the phosphorylation of proteins and for signal transduction when there are other much more rich reserves of P that could be utilised (for example nucleic acids or phospholipids), this seems unlikely.

PEPC proteins were identified in the root membrane associated proteome, soluble proteome as well as the root phosphoproteome, to have increased in abundance in response to Pi starvation. BrPEPC1,2,3 proteins (A0A3P5ZNL1, A0A3P5YR48, A0A397Y6V5) increase in abundance in response to Pi starvation in the root proteome, identified in both the membrane associated and the root soluble proteome. BrPEPC2 and BrPEPC3 phosphorylated at S11 (A0A3P5YR48, A0A3P5Z9C2) was also identified to have increased in abundance in the phosphoproteome, they are two out of the six phosphopeptides that increased in abundance during Pi starvation. It is hard to judge whether the increased abundance detected in the phosphoproteome is due to increased phosphorylation of the phosphopeptide or due to a general increased abundance of the protein, as there could just be a consistent phosphorylation at S11. The increase in PEPC proteins identified in all proteomes indicates that it is a highly abundant protein that is responsive to Pi starvation, but the responsiveness of the phosphorylation to Pi availability is unclear.

PEPC kinase proteins are also increased in abundance in the membrane associated proteome, but not in the other root proteomes, in response to Pi starvation. PEPCK proteins are calcium independent kinases involved in PEPC phosphorylation [Nimmo, 2000, Vidal and Chollet, 1997], therefore we can hypothesise that the PEPCK proteins we see in our datasets could be responsible for the PEPC phosphorylation seen in the phosphoproteome detected to increase in response to Pi starvation, but functional characterisation would be needed to confirm this. In *Arabidopsis* it has already been reported that PEPC proteins are responsive to Pi starvation conditions, and suggests that the phosphorylation of PEPC1 contributes to metabolic adaptations of *Arabidopsis* to Pi stress to improve Pi efficiency [Gre-

gory et al., 2009].

The phosphopeptides which decreased in abundance in respone to Pi starvation had an enrichment in a variety of phosphorylation motifs, (-SP-, -R–S-, P-SP, and TP)(Table 5.3). These motifs can give an indication of what kinases acted on the peptides to cause the phosphoylation during sufficient Pi conditions compared to Pi starved conditions, as well as indicate the phosphatases that function to dephosphorylate the proteins during Pi starvation. -SP- and -TP- motifs are commonly targetted by MAPK, SnRK2, RLK, AGC kinase, cyclin-dependent kinases, calcium dependent protein kinases, and STE20-like kinase. -R–S- motifs are also commonly targetted by MAPK [van Wijk et al., 2014]. Knowing what kinases are likely to play a role in the phosphorylation events that change in response to Pi stress could give an indication of what kinase proteins activity changes in response to Pi stress, and give an idea of the different signalling events and pathways that occure. But I think in this case it is not that informative as the motifs enriched and what they can be targeted by pretty much involves every type of kinase there is.

5.4 Conclusion

The aims of this chapter were to identify what proteins change in response to Pi starvation in the roots of *B. rapa* plants. The root proteome and phosphoptoteome was determined to change significantly in response to Pi starvation. Various proteins suspected to be involved with improving Pi efficency were identified to significantly increase in abundance, such as Pi transporters, ATPase, PAP, RNase and lipid remodelling proteins, which could all potentially be involved with improving the accessibility and uptake of Pi. Further functional studies would be required to understand the specific roles these proteins play in improving Pi efficiency during Pi deficiency in *B. rapa*. The signalling events in the roots of *B. rapa* appear to change in response to Pi starvation indicated by the changes in the roots phosphoproteome. There is a large amount of phosphopeptides identified to have decreased in response to Pi starvation, while few are increased, indicating different proteins are involved in potential signalling pathways during a phosphate starvation response in *B. rapa* roots, however further work is needed to understand what the functions of the proteins and the phosphorylation events are.

Chapter 6

Quantitative Transcriptomic and Proteomic Analysis of Phosphate Starved *B. rapa* Shoots

6.1 Introduction

Previously in this thesis more emphasis has been focussed towards identifying the root responses to Pi starvation. Roots are an important target in understanding plants phosphate starvation response (PSR). Roots are the interface between the plant and the environment in which they absorb Pi, they interact with the local area to promote a beneficial environment to improve Pi accessibility and uptake efficiency, and are essential in the sensing of exogenous Pi levels [Svistoonoff et al., 2007]. Root responses to Pi deficiency allow the adaptation of plants to improve Pi uptake efficiency and to improve the accessibility of Pi to be taken up. Such as utilising transporters to improve Pi uptake [Chang et al., 2019], or PAP proteins to improve to availability of Pi from Po sources [Wang et al., 2009] resulting in improved Pi efficiency. Plant shoot material also plays a role in adapting to the changes in nutrient availability. A PSR in the shoots results in the plants improving their Pi utilisation efficiency. Previous work looking at shoot responses to Pi stress is largely carried out in *Arabidopsis* and rice plants, and summarizes that shoots respond to Pi starvation by increased Pi recycling, increasing Pi remobilisation from storage

and source tissues to maintain Pi homeostasis, reducing photosynthesis, anthocyanin accumulation, lipid remodelling, and increased surger concentration [Zhang et al., 2014].

The remobilisation of Pi from source to sink material within the plant occurs during Pi limiting conditions, therefore systemic communication is required between the root and shoot. Less work has been documented on *Brassicas* molecular responses to Pi starvation in shoots. Analysing at transcriptomics and proteomic responses seems to be a good place to start to identify targets which can be implicated in improving Pi efficiency, which could then be utilised to further improve plants Pi deficiency tolerance.

The transcriptomic changes observed in plants can reveal the molecular mechanisms of their PSR. But mRNA information is not fully complete as the down stream proteins are not identified, and the gene expression doesn't necessarily determine protein abundance, nor protein function. As the complexity of the samples increases the interpretation of the data also increases in complexity. Genomic information within a plant has a relatively low complexity, *B. rapa* contains an excess of 40,000 protein coding genes, and these protein coding genes are responsible for the production of thousands of mRNA transcripts, with a single gene producing multiple transcripts with potentially different splice variants, these transcripts then lead to the translation to proteins. Many down stream events then play a significant role in determining protein function, such as; localisation, protein-protein interactions, co-factors, and a plethora of PTM of which phosphorylation is the most common and which also commonly plays a role in signalling pathways [Khoury et al., 2011]. Looking at the transcriptomic, proteomic and phosphoproteomic responses to Pi starvation can give a large breadth of data to analyse, and to give a broad idea of the PSR in *B. rapa* shoots, with various levels of complexity with each method having their advantages and disadvantages.

Transcriptomic analysis allows a high sensitivity for a better coverage of transcripts to be identified. Allowing gene expression levels to be determined and to identify differentially expressed genes whose protein products are not always easily identifiable during proteomic analysis. A downside is the transcript abundance does not always correlate with the protein abundance or protein activity, and down stream information such as PTM will be missed, this is due to the complex regualtory mechanisms involved after transcription, such as PTM, RNA silencing, abberant splicing forms and synthesis of protein inhibitors all potentially effecting protein abundance or activity [Zapalska-Sozoniuk et al., 2019].

Proteomic analysis can allow a more accurate abundance of the final gene products in the form of proteins to be measured. Useful tools such as Co-immunoprecipitation can be utilised to identify protein interactions, and PTM can be identified. A downside is that not all proteins are easily identifiable by proteomic analysis, and low abundant proteins can be overlooked in favour of those of higher abundance. Also PTM will be missed unless they are specifically included in the database search. A well annotated proteome is also required to identify proteins present. The sensitivity is also not as high as with RNA-seq analysis.

Phosphoproteomic analysis allows the identification of phosphorylated peptides after phosphopeptide enrichment. Phosphorylation identification can allow the identification of signalling events. The downside of phosphoproteomic analysis is that the interpretation of the data is often difficult, as even though identifying the phosphopeptide can indicate a signalling event through phosphorylation, actually piecing the signalling pathway together, identifying the kinase proteins responsible for the phosphorylation and determining how the modification effects the function of the protein or the role it plays in a signalling pathway is difficult.

Overall these omic analytic techniques are a powerful tool to give a good starting point to identify candidates involved in plant responses to Pi stress that improve Pi efficiency. Understanding how these candidates improve Pi efficiency could then allow them to be utilised to further improve a plants tolerance to Pi deficient conditions. These candidates could be utilised through over expression lines to improve plant growth, or by screening for them in breeding populations to creating breeding lines with an enhanced presence of the candidates to improve in Pi efficiency. However, further analysis such as biochemical characterisation and functional assays of the protein candidates is required to get a clearer idea of how the proteins improve a plants Pi efficiency.

6.1.1 Aims

The aim of this chapter was to determine how the shoots of *B. rapa* plants respond to Pi starvation. Analysing changes in gene expression and protein abundance in response to Pi starvation could allow the identification of proteins involved in improving Pi efficiency during Pi deficient conditions, these could be potential targets to further improve plants Pi efficiency in the future. Identifying signalling proteins that change in response to Pi deficient conditions could indicate proteins involved in phosphate starvation responsive signalling pathways in *B. rapa* shoots. To achieve this aim I induced a PSR in *B. rapa* R500 plants and used the shoot material

to;

- Quantitatively analyse changes in the shoot transcriptome.
- Quantitatively analyse changes in the shoot proteome.
- Quantitatively analyse changes in the shoot phosphoproteome.

6.2 Results

In order to analyse the transcriptomic and proteomic changes in *B. rapa* shoots in response to Pi starvation a PSR had to be induced. *B. rapa* R500 plants were grown hydroponically until 20 days old, then a PSR was induced with Pi deficient media for 11 days. Shoot material was collected and the shoot RNA and proteins were extracted. RNA was quality checked and then sent to Novogene UK for sequencing to use for RNA-seq analysis. Proteins were extracted for proteomic analysis using MS, 1 mg of protein extract was used for a phosphopeptide enrichment for phosphoproteomic analysis using MS (Fig. 6.1).

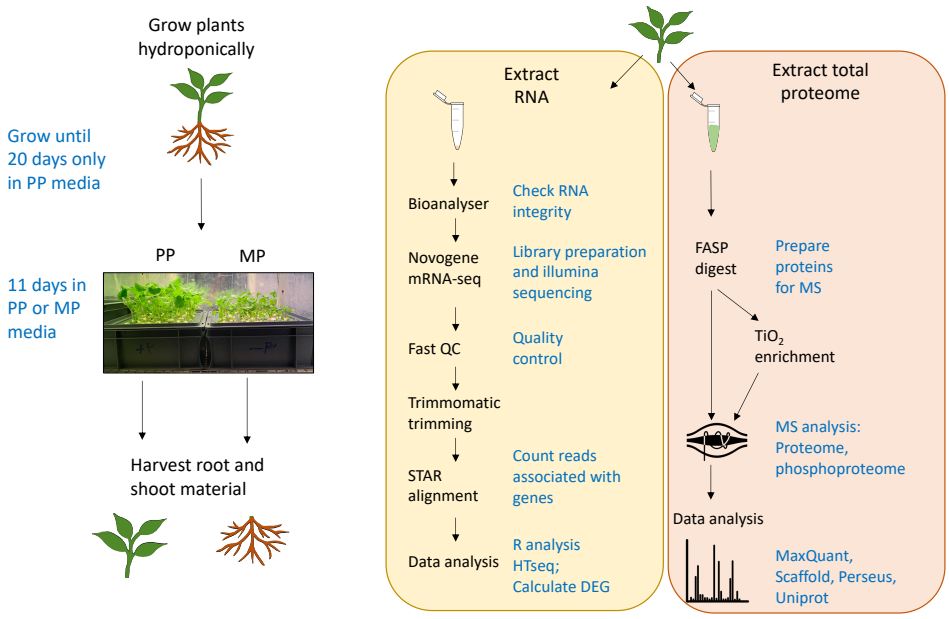

Figure 6.1: Preparation of *B.rapa* shoot samples: *B. rapa* R500 plants were grown hydroponically in Pi sufficient (PP) media until 20 days old, after which half were transferred into Pi deficient (MP) media, while the other half were maintained in PP media for 11 days before the shoot material was harvested. Shoot RNA was extracted and sent for sequencing to be used for RNA-seq analysis. Shoot proteins were extracted then prepared for MS by a FASP digest, some samples were sent straight to MS for total proteome MS analysis, the rest was enriched for phosphopeptides using TiO_2 affinity binding before being used for phosphoproteomic MS analysis. The data was analysed using a variety of software and web tools.

6.2.1 PSR in *B. rapa* leads to a significant change in shoot transcriptome with an increase in gene expression related to cellular responses to Pi starvation

Gene expression changes in response to Pi starvation with various Pi starvation induced (PSI) genes getting activated for the plants PSR to better improve their Pi efficiency. RNA from the shoot material of plants with an induced PSR were extracted to compare against control samples. Library preparation and sequencing was performed by Novogene UK, three biological replicates per condition were used for transcriptomic analysis.

After sequencing, reads were trimmed and mapped to the *B. rapa* genome, the genome and annotation used was *B. rapa* R500 version 52010 from CoGe [Lyons and Freeling, 2008]. STAR RNA-seq alignment tools [Dobin et al., 2013] was used for sequence alignment, more than 90 % of raw reads were uniquely mapped to the *B. rapa* genome when checked using MultiQC 1.11 [Ewels et al., 2016], and between 18 and 21 million uniquely mapped reads were identified in each sample.

33,025 genes were identified, these were filtered so they had to be expressed in all 3 repeats of at least one growth conditions, leaving 28,671 expressed genes identified for further analysis.

To determine if there was a difference in gene expression between Pi conditions, analysis of was performed using the R package DESeq2 [Love et al., 2014]. A PCA was performed and revealed a separation of the samples with a component 1 variance of 86 % (Fig. 6.2A) while the samples within replicates group together. The differentially expressed genes (DEG) were calculated using DESeq2 with a Wald's test, with a p-value <0.05 and a $\log_2$ fold change >1. A volcano plot was used to represent the DEG, plotting $\log_2$ fold change against the -log p-value (Fig. 6.2B). There were 5,279 of DEG identified, 3,456 genes increased expression in response to Pi starvation, and 1,733 had decreased expression in response to Pi starvation.

The high number of DEG makes analysing them individually and displaying them all unmanageable, therefore GO-term enrichment analysis was performed to see if any processes or functions are significantly enriched in response to Pi starvation. The genes that increased in expression during Pi starvation show an enrichment of a number of biological processes including cellular response to P starvation, P ion transport, Po metabolic process, carbohydrate metabolic process, and flavonoid biosynthesis (Fig. 6.3A). The genes that increased in expression had an under-enrichment in intracellular protein transport, proteolysis, translation and membrane organiza-

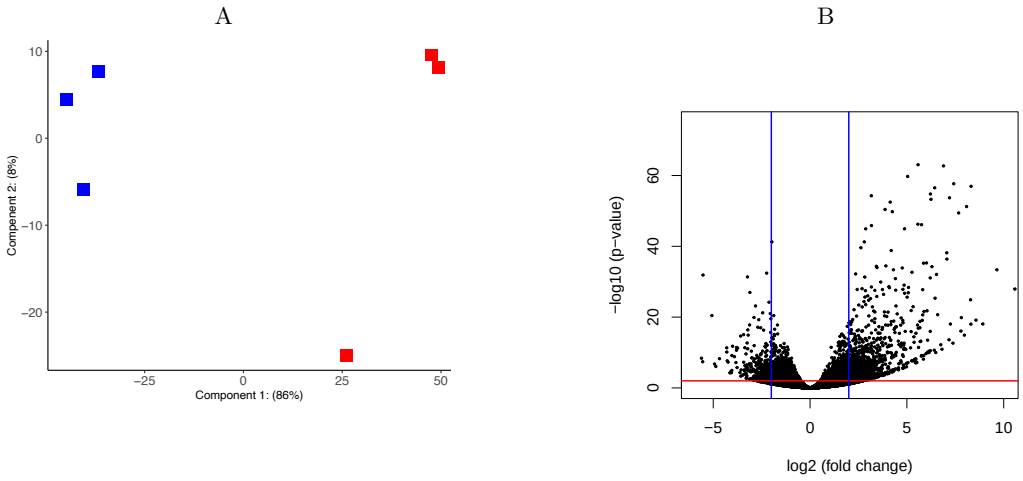

Figure 6.2: Quantitative analysis of *B. rapa* transcriptome in response to Pi starvation: The transcripts identified in *B. rapa* shoots analysed using DESeq2. A) PCA plot, Pi starved samples highlighted in red, control samples in blue. B) Volcano plot, $\log_2$ fold change in gene expression (Pi starved samples minus control samples) plotted against -log p-value determined by Wald test. Red line is the P-value cut off < 0.05, blue lines represent FC cut off $\log_2$ 1, identifying the significantly DEG.

tion. Molecular functions that were enriched during Pi starvation included acid phosphatase activity, active TM transporter activity and G3P 2-O-acyltransferase activity, while there is a decreased enrichment in RNA binding and structural constitution of ribosomes (Fig. 6.3C). GO term enrichment shows that shoot response to Pi starvation leads to an increase in gene expression that could be matched with phosphate starvation responsive genes that improve Pi efficiency.

The genes which decreased in expression in response to Pi starvation had an enrichment in a variety of biological processes including proteolysis, phospholipid translocation and RNA polymerase transcription (Fig. 6.3B). Molecular functions which were enriched included phosphatidylinositol-3-phosphate binding, lipid transporter activity, kinase activity, and proteosome activating activity (Fig. 6.3D). Indicating that *B. rapa* have decreased gene expression in various processes in response to Pi deficiency, this could be a part of its PSR to improve Pi efficiency by moving resources away from certain processes when Pi is lacking.

To classify the types of proteins from the genes which change in expression during Pi starvation PANTHER 16.0 was used to assign Protein classes and to perform enrichment analysis. Of the 3,456 genes that increase in expression during Pi starvation only 1,609 were assigned a protein class, the distribution of the classes designated are represented in Fig.6.4A. The most common PC identified was metabolite interconversion enzymes, consisting of 18.8% of all the identified genes that increased in

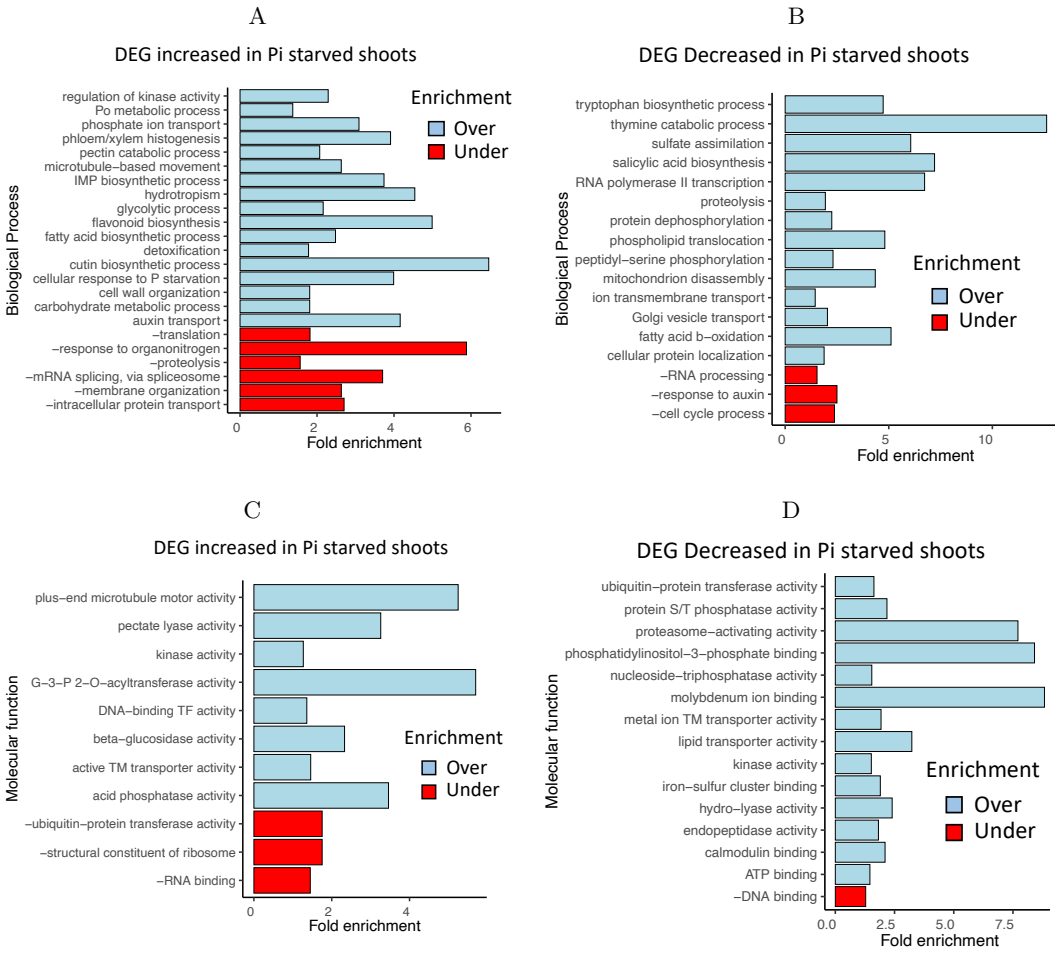

Figure 6.3: *B. rapa* shoot transcriptome GO-term enrichment in response to Pi starvation: GO-term analysis of shoots significantly DEG using PANTHER 16.0. Displaying terms FE which are significantly enriched using a Fisher exact test with a cut off FDR < 0.05, terms over enriched are coloured in blue, terms that are under enriched are coloured in red. A, B) Biological processes GO-term enrichment. C, D) Molecular functions GO-term enrichment. A, C) Genes increased in expression during Pi starvation. B, D) Genes decreased in expression during Pi starvation.

expression, it includes hydrolase and lyase enzymes which were also enriched. The other enriched PC identified was gene-specific transcriptional regulators.

Of the 1,733 genes that displayed a decrease in expression in response to Pi starvation 46% of them did not have a panther protein classification (Fig. 6.4B). The metabolite interconversion enzyme protein class was also enriched but the enzymes included which were enriched were transferase, oxidoreductase and hydrolase enzymes, in contrast to just hydrolase enzymes that were enriched in the genes that increased in expression. The genes that decreased also showed an enrichment in protein modifying enzymes, transporter proteins and transmembrane signal recep-

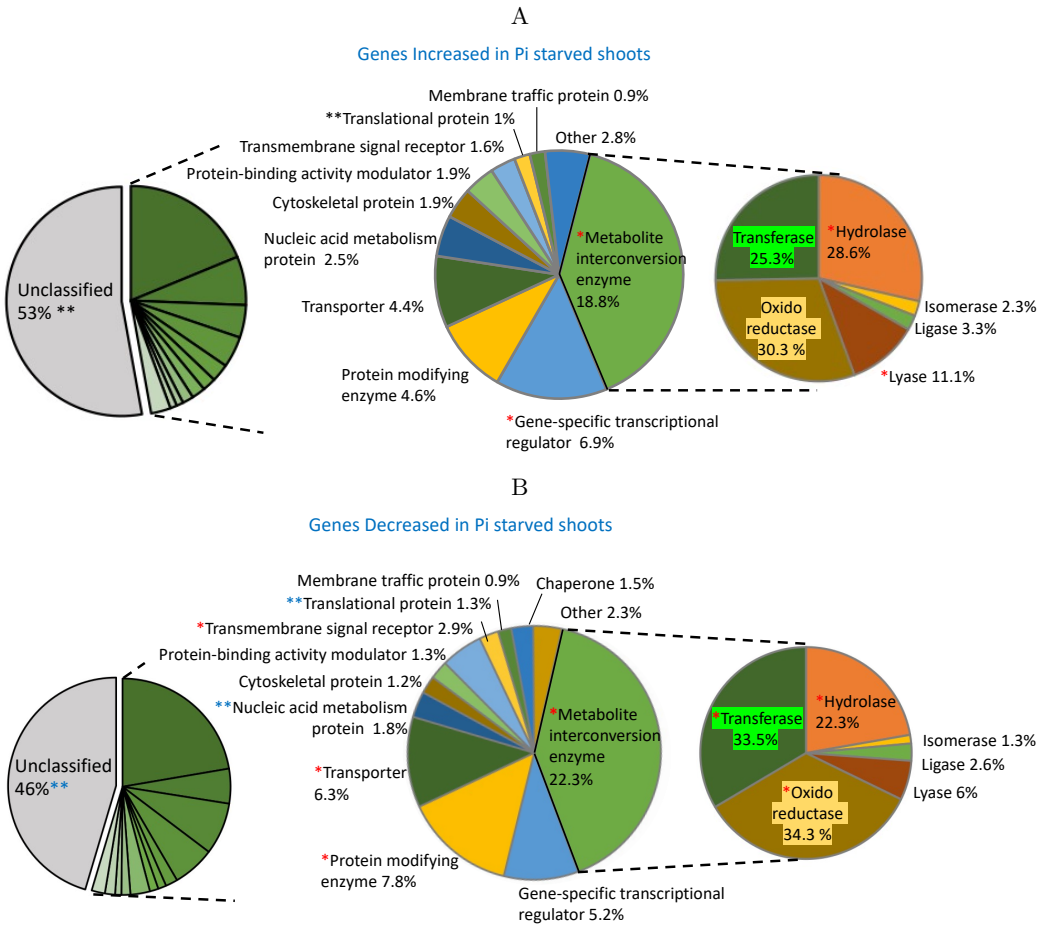

Figure 6.4: Classification of *B. rapa* transcriptome response to Pi starvation: The DEG in response to Pi starvation protein classifications was determined using PANTHER16.0, enrichment analysis of the protein classes performed using Fisher exact test with a cut off FDR < 0.05, * indicate classes over-enriched, ** denote classes under-enriched. A) PC of genes with increased expression during Pi starvation, 53% were unclassified so the proportion of the PC for the genes which had a classification are represented. Showing the proportion of the enzymes that make up metabolite interconversion enzymes. B) PC of genes with decreased expression during Pi starvation, 46% were unclassified so the proportion of the PC for the genes which had a classification represented. Showing the proportion of the enzymed that make up metabolite interconversion enzymes.

tor proteins. Protein classification shows that there are significant enrichments in certain protein classes that change significantly in response to Pi starvation.

Due to the high number of DEG that were identified, only a few of interest which could be involved in improving plants Pi efficiency as a part of a PSR are displayed in Table 6.2. 12 PAP genes and 2 diphosphatase genes were detected to have a large increase in expression during Pi starvation, while 2 PAP genes decreased in expression, despite an enrichment for acid phosphatase activity during Pi starvation. Genes involved in the GO term cellular response to P starvation, which was enriched

in response to Pi starvation, include some of the phosphate transporters and SPX-domain containing proteins which are suspected of playing a role in Pi sensing and signalling, show increased expression during Pi starvation. RNase genes and genes involved in lipid remodelling also showed an increased expression during Pi starvation.

Transcriptomic analysis of *B. rapa* shoots indicates a significant response in the gene expression levels of shoots during Pi starvation. Various genes have a significant change in expression when in Pi deficient conditions. With an increase activation of genes involved in cellular response to Pi starvation expected to better help the plant to tolerate low Pi conditions as a part of their PSR.

Table 6.1: *B. rapa* shoot DEG in response to Pi starvation: A subset of the DEG which were identified in the shoots in response to Pi starvation suspected to play a role in Pi tolerance. Gene name from the CoGe database used for the sequence alignment, the corresponding *B. rapa* Chifu homologue used for the enrichment analysis, protein name, and the -log P-value for the $\log_2$ FC based of a wald's test. * Indicate proteins that were uncharacterised so named from the closest *Arabidopsis* homologue.

Gene	Chifu homologue	Protein name	Log_2 FC	-log P-value
Phosphatase				
BraA03g59110R	Bra017755	PAP6	8.9	18.1
BraA05g28330R	Bra022244	PAP17	8.3	57.0
BraA07g03270R	Bra039919	PAP5	8.3	18.0
BraA09g42140R	Bra006951	PAP22	5.2	15.9
BraA01g27240R	Bra021274	PAP17	4.8	33.9
BraA03g43640R	Bra013072	PAP10	4.7	12.1
BraA01g08550R	Bra013843	PAP24	2.8	31.3
BraA04g18720R	Bra034307	PAP12	2.4	8.9
BraA09g59990R	Bra026890	PAP1	2.0	18.4
BraA09g11230R	Bra037302	PAP10	1.4	1.9
BraA05g37160R	Bra029624	PAP15	1.3	2.5
BraA01g33490R	Bra040363	PAP15	1.1	1.9
BraA09g42880R	Bra007016	Inorganic diphosphatase 4	4.3	33.4
BraA04g06840R	Bra039808	Inorganic diphosphatase 4	4.3	49.8
BraA05g39730R	Bra040607	Inositol-phosphate phosphatase	1.5	12.1
BraA08g03570R	Bra014262	Sucrose-phosphatase 1	6.2	54.8
BraA04g07990R	Bra033441	Sucrose-phosphate phosphatase	1.8	4.8
BraA04g24740R	Bra017287	Sucrose-phosphatase	1.4	3.9
BraA02g36950R	Bra026550	PAP9	-1.4	3.6
BraA03g43600R	Bra013069	PAP10	-1.1	4.1
BraA07g40750R	Bra018135	H(+)-exporting diphosphatase	-1.8	1.4
BraA10g26620R	Bra009396	Inorganic diphosphatase 6	-1.4	10.0
Lipid metabolism				
BraA02g09730R	Bra020104	MGDG synthase 2	7.2	20.4
BraA09g10650R	Bra037199	MGDG synthase 3	7.1	108.1
BraA02g31500R	Bra035967	GDPD3-like	5.2	21.9
BraA09g19930R	Bra027481	GDPD3	4.1	14.2
BraA10g27360R	Bra009316	GDPD6	3.2	22.9

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Table 6.1 – *Continued from previous page*

Gene	Chifu homologue	Protein name	Log_2 FC	-log P-value
BraA07g18970R	Bra028485	GDPD2	2.7	5.0
BraA02g18060R	Bra034041	GDPD1	-1.7	8.0
BraA05g04060R	Bra004724	Phospholipase A1	4.97	6.9
BraA03g56810R	Bra037029	UDP-sulfoquinovose synthase 1 *	3.35	27.6
BraA03g01360R	Bra005694	Sulfoquinovosyl transferase SQD2 *	2.75	9.6
BraA10g15740R	Bra002626	Phosphoinositide phospholipase C	3.0	12.3
BraA10g15730R	Bra002627	Phosphoinositide phospholipase C	2.3	15.2
Pi signalling				
BraA03g23100R	Bra000373	SPX domain-containing protein 3	8.6	19.2
BraA04g30630R	Bra040324	SPX domain-containing protein 3	7.7	49.4
BraA03g09680R	Bra006543	SPX domain-containing protein 1	5.8	84.0
BraA02g09590R	Bra020088	SPX domain-containing protein 1	5.6	113.6
BraA03g24840R	Bra000539	SPX domain-containing protein 2	2.1	11.8
BraA09g13500R	Bra036635	SPX domain-containing protein	-1.1	10.8
Transporter				
BraA02g19450R	Bra038357	PHO1 homolog 1*	7.1	38.2
BraA05g21250R	Bra034426	SPX domain-containing transporter	6.2	16.0
BraA04g06060R	Bra014798	inorganic phosphate transporter 1-7 *	5.4	7.1
BraA06g41530R	Bra033676	inorganic phosphate transporter 1-3 *	5.3	32.7
BraA07g28920R	Bra004017	PHO1 homolog 1*	4.7	11.6
BraA09g19970R	Bra027487	inorganic phosphate transporter 1-3*	4.2	9.8
BraA07g32190R	Bra004334	PHO1 homolog 1*	3.7	27.8
BraA09g19960R	Bra027486	inorganic phosphate transporter 1-6*	3.6	3.6
BraA02g31530R	Bra035976	inorganic phosphate transporter 1-6*	2.7	2.1
BraA03g17300R	Bra022925	inorganic phosphate transporter 1-5*	2.5	10.5
BraA01g23010R	Bra023727	PHO1*	2.5	2.7
BraA05g07370R	Bra005069	inorganic phosphate transporter 1-4*	2.1	16.5
BraA09g59430R	Bra026864	PHO1 homolog 3*	-2.5	3.8
BraA06g10090R	Bra019689	PHO1 homolog 3 *	-2.4	6.7
BraA09g36960R	Bra024691	PHO1 homolog 8 *	-2.3	4.1
BraA09g59740R	Bra026919	Phospholipid-transporting ATPase	-1.6	3.6
BraA01g20120R	Bra035422	Phospholipid-transporting ATPase	-1.3	3.0
BraA02g22350R	Bra008042	Phospholipid-transporting ATPase	-1.2	6.1
BraA09g57480R	Bra030991	Phospholipid-transporting ATPase	-1.1	4.6
BraA02g37510R	Bra026515	Phospholipid-transporting ATPase	-1.0	2.0
Misc				
BraA10g23590R	Bra008792	Chalcone synthase 1*	8.08	51.2
BraA03g06590R	Bra006224	Chalcone synthase 3*	7.81	19.9
BraA10g27400R	Bra009312	Flavonoid 3'-monooxygenase *	7.07	36.4
BraA08g32540R	Bra030737	PEPC Kinase 1	7.42	57.7
BraA05g30600R	Bra001575	PEPC3	4.8	20.7
BraA05g03900R	Bra004710	PEPC2	1.6	5.1
BraA08g02420R	Bra030945	PEPC1	1.6	12.4
BraA06g27480R	Bra024410	PEPC kinase 2	-2.0	1.6
BraA08g29700R	Bra016708	Ribonuclease T(2)	5.6	11.4
BraA02g36760R	Bra026570	Ribonuclease T(2)	2.7	7.6

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Table 6.1 – *Continued from previous page*

Gene	Chifu homologue	Protein name	Log_2 FC	-log P-value
BraA09g36610R	Bra024724	1-phosphatidylinositol 4-kinase	-1.3	10.6
BraA04g31380R	Bra039280	1-phosphatidylinositol 4-kinase	-1.2	2.8

6.2.2 PSR in *B. rapa* leads to a significant change in the shoot proteome with an increased abundance of phosphatase proteins

After seeing the changes in the shoot transcriptome in response to Pi starvation I will also show the changes in the shoot proteome. Identifying proteins which change in abundance in response to Pi starvation can supply some insight into the shoot responses to phosphate stress. The shoot material of 31 day old *B. rapa* was harvested after a PSR had been induced, and the total soluble proteome was extracted to be identified using MS for quantitative analysis with three replicates.

After the MS analysis a total of 151,263 MS spectra were recorded, which were assigned to 2,626 proteins. These proteins were filtered for potential contaminants, false detection and low confidence proteins, and further filtered so the proteins had to be present in the 3 replicates of at least one Pi condition to be kept for analysis. This left 1,249 proteins identified in the shoot proteome.

Using the LFQ intensity values the identified proteins were analysed to determine changes in the shoot proteome in response to Pi starvation. A PCA was performed (Fig. 6.5A) and shows a clear separation between sample conditions, with a component 1 variance of 65.8% while samples of the same condition grouped together. Indicating a difference in the proteome of *B. rapa* shoots in response to Pi starvation.

A student two-sample t-test was performed on the proteins LFQ intensities to identify proteins that are significantly changed in abundance in response to Pi starvation, using an FDR of 0.05 and log_2 FC of 1. A volcano plot was made to represent this data, plotting log_2 FC difference, Pi starved sample values minus control sample values, against -log p-value, points in red show the significantly DAP (Fig. 6.5B). Of the 1,249 proteins identified 429 were significantly changed in abundance, 139 were increased in abundance in response to Pi starvation while 290 were decreased in abundance. The differentially abundant proteins were normalised using Z-score and used to create a heat map (Fig. 6.5C). The sample replicates cluster together

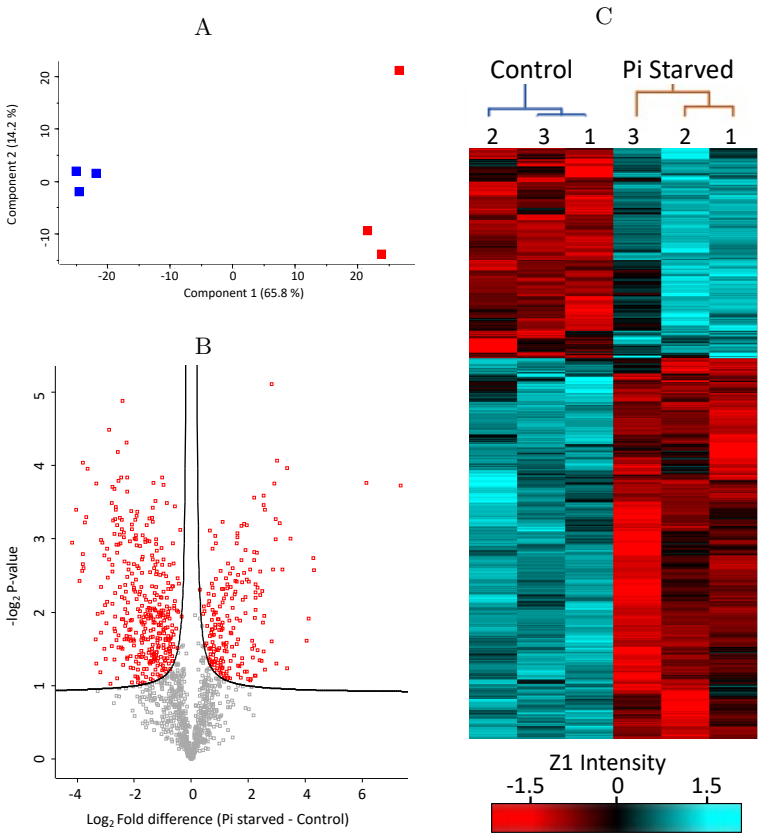

Figure 6.5: Quantitative analysis of *B. rapa* shoot proteome in response to Pi starvation: The proteins identified in the shoot samples were analysed in Perseus using LFQ intensity values. A) PCA plot of shoot proteins. Pi starved samples are highlighted in red, control samples are highlighted in blue. B) Volcano plot of shoot proteins, using a student two sample t-test to identify proteins significantly changed in abundance, $\log_2$ fold change is plotted against -log P-value. Red points show the 566 proteins which are significantly changed in abundance in response to Pi starvation, FDR:0.05 s0:0.1. C) Heat map for the 566 significantly changed proteins between PP and MP conditions. LFQ log2 intensity values normalised using the Z-score for graphical representation.

based on Pi conditions and there is a distinction between the proteins which increased in abundance verses the proteins that decreased in abundance, indicating a clear difference in *B. rapa* shoot proteins during Pi starvation.

GO-term enrichment analysis to determine changes in predicted processes and functions of proteins that change in response to Pi starvation was performed using PANTHER 16.0 with a Fisher exact test and a FDR correction < 0.05. Proteins that increased in abundance in response to Pi starvation had numerous biological processes enriched, such as UTP biosynthetic regulation, abscisic acid biosynthesis, proton transmembrane process, and carbohydrate metabolic process as some with the highest FE (Fig. 6.6A). There were only a few molecular functions enriched; acid phosphatase activity with hydrolase activity and functions involving oxidoreductase

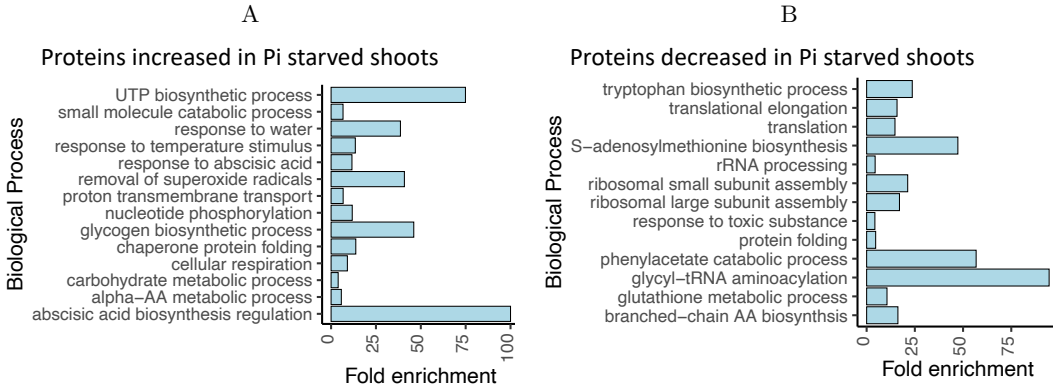

Figure 6.6: *B. rapa* shoot proteome GO-term enrichment in response to Pi starvation: GO-term analysis of proteins that significantly changed in abundance using PANTHER16.0 with a fisher exact test and FDR correction of <0.05, displaying FE of significantly enriched terms. A) Biological processes of proteins increased in abundance in response to Pi starvation B) Biological processes of proteins decreased in abundance in response to Pi starvation.

activity acting on superoxide radicals as acceptor, but these functions indicate a change in the proteins present in response to Pi deficiency which could help improve Pi efficiency.

The proteins which decreased in abundance in response to Pi starvation showed an enrichment in a number of processes including translation, ribosomal assembly and ammino acid biosynthetic processes (Fig. 6.6B). Molecular functions showed an enrichment of structural constitute of ribosomes, and RNA Binding, with a large proportion of the proteins significantly decreased being ribosomal proteins.

The Panther protein classification was used to determine the proportion of the different classes of proteins identified to have changed in abundance in response to Pi starvation, to see if there are certain classes that are more responsive to Pi starvation than others. The proteins that increased in abundance in response to Pi starvation were mostly classed as metabolite interconversion enzymes at 46.5%, of which hydrolase, lyase and oxidoreductase enzymes were enriched (Fig. 6.7A). Of the proteins that decreased in abundance in response to Pi starvation 34% were translational proteins, which was an enriched class of protein, and includes ribosomal proteins (Fig. 6.7B). Chaperone and metabolite interconversion enzymes were enriched, of which transferase and lyase enzymes were enriched. protein classification Indicates a significant difference in the proteins that changed in abundance in response to Pi starvation.

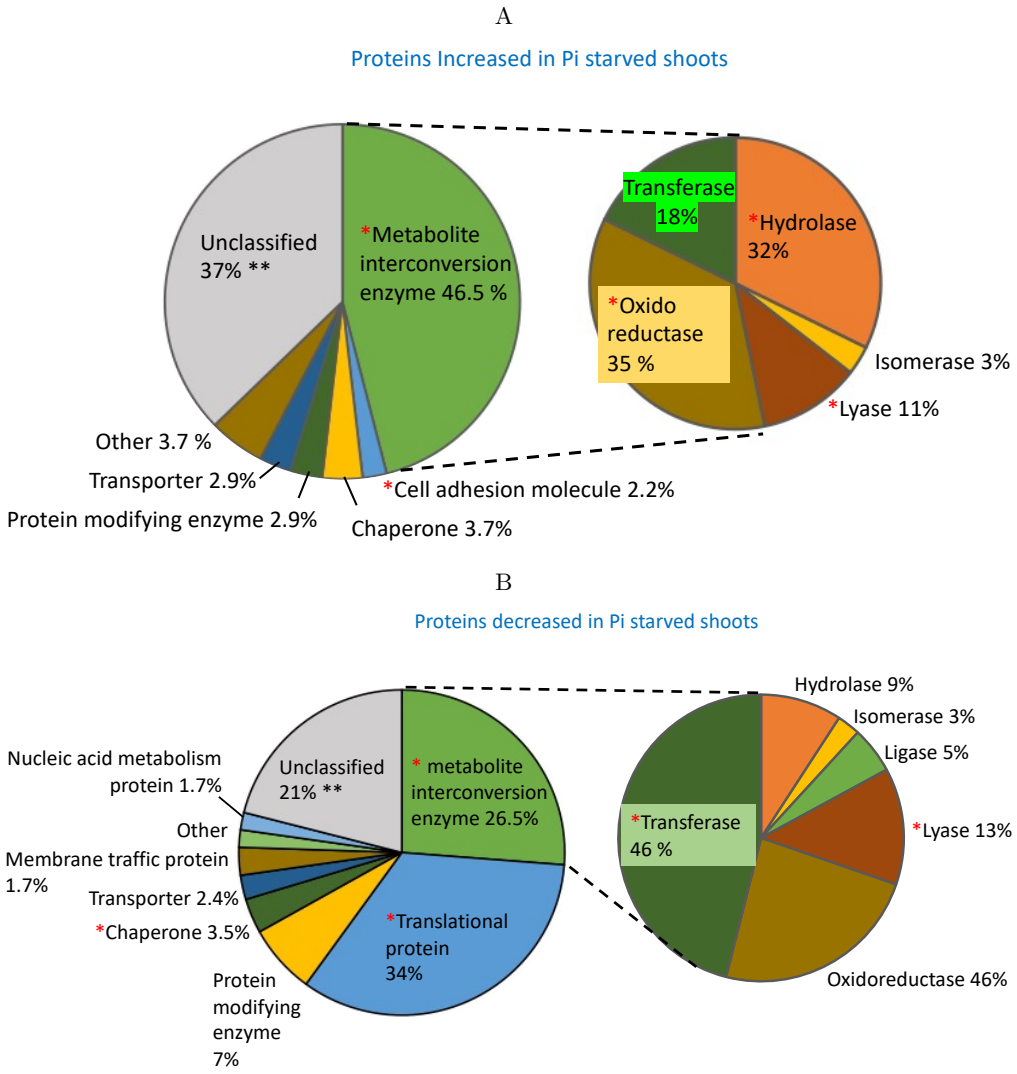

Figure 6.7: Classification of *B. rapa* shoot proteome response to Pi starvation: The proteins that changed in abundance in response to Pi starvation protein classifications were determined, and enrichment analysis performed using PANTHER16.0 with a fisher exact test and FDR correction of <0.05. A) Proteins that increased in abundance in response to Pi starvation protein classes. B) Proteins that decreased in abundance in response to Pi starvation protein classes. * Denotes PC which were significantly enriched. ** Denotes PC which were significantly under enriched.

A subset of the proteins that significantly changed are represented in Table 6.2. To make the table more manageable the proteins that are expected to be involved improving Pi efficiency and are part of enriched processes identified are displayed. Two PAPs and some diphosphatase and inositol phosphatases were identified to have increased in abundance in response to Pi starvation. The increase in these proteins could be a part of the shoot PSR to improve Pi efficiency when in Pi deficient conditions.

Table 6.2: *B. rapa* shoot proteins that significantly change in abundance in response to Pi starvation: A subset of proteins identified which change in abundance are displayed and grouped based on protein groups or processes enriched in response to Pi starvation which may have a role in improving shoot Pi efficiency. Protein ID and names come from uniprot, chiifu homologue from BLAST, $\log_2$ FC, and -log p-value calculated with student t-test.

Protein ID	Chifu homologue	Protein name	Score	$\log_2$ FC	-log P-value
Phosphatase					
A0A3P6BYS0	Bra039808	Inorganic diphosphatase	4.1	2.9	3.3
A0A3P5Y9A6	Bra037302	PAP 10	12.0	2.9	2.6
A0A3P5YTG9	Bra026890	PAP1	8.7	2.3	2.8
A0A3P5ZFV4	Bra006536	NAD(+) diphosphatase	82.0	1.7	3.2
A0A3P6CXN1	Bra009396	Inorganic diphosphatase	78.4	1.1	1.2
A0A3P5YVH4	Bra040607	Inositol-phosphate phosphatase	33.9	1.0	1.3
A0A3P5YF97	Bra023165	Inositol-1-monophosphatase	5.3	-2.5	2.3
A0A397ZJ45	Bra014569	Serine/threonine-protein phosphatase	20.9	-1.8	2.3
A0A397XWT7	Bra029552	inositol monophosphatase*	4.0	-1.1	1.7
Transport					
A0A397ZZI1	Bra040512	V-type proton ATPase subunit G	3.2	4.3	2.6
A0A3P5ZSU6	Bra019275	V-type proton ATPase subunit G	299.3	1.9	2.2
A0A397KYN2	Bra017671	Complex I-B22 NADH dehydrogenase	3.1	1.3	1.1
A0A397YHM9	Bra030675	RanBD1 domain-containing protein	47.9	1.1	1.4
A0A397Y9X0	Bra007415	vATPase subunid D*	4.5	1.2	1.5
Protein folding					
X4YCX7	Bra010728	Peptidyl-prolyl cis-trans isomerase (PPIase)	10.0	6.1	3.8
A0A397Z964	Bra018315	Tubulin-specific chaperone A	23.2	2.1	1.5
A0A397XYB3	Bra032797	CCT-epsilon	82.81	-1.91	2.9
A0A3P5XW25	Bra037296	Peptidyl-prolyl cis-trans isomerase (PPIase)	110.85	-1.27	2.0
Carbohydrate matabolic process					
A0A3P6B3V5	Bra014340	Fructose-bisphosphate aldolase	19.9	3.2	2.6
A0A3P5Z7R1	Bra005269	1,4-alpha-glucan branching enzyme	13.2	3.1	3.2
A0A397Z166	Bra009763	Probable 6-phosphogluconolactonase	4.6	3.0	4.1
A0A397XKW7	Bra002289	Sucrose-phosphate synthase	7.2	1.8	3.2
A0A3P6BXF6	Bra004012	Sinigrinase	3.2	2.6	3.4
A0A397YME2	Bra030799	Phosphoglycerate mutase	72.4	1.6	3.1
A0A3P5ZN96	Bra001470	Phosphoglycerate kinase	5.6	1.1	3.2
A0A3P6CK72	Bra002221	Glucose-1-phosphate adenylyltransferase	27.1	1.1	1.8
Defence/Detox response					
A0A397Z3N9	Bra024879	Bet_v_1 domain-containing protein	43.1	4.0	1.6
A0A3P6B4Q6	Bra028091	Expansin-like EG45 domain-containing protein	5.5	3.0	3.0
A0A3P6DEP2	Bra002133	Superoxide dismutase [Cu-Zn]	4.3	1.5	1.8
A0A3P6CJA6	Bra034394	Superoxide dismutase [Cu-Zn]	70.4	1.2	1.5
A0A3P5Y9S3	Bra031642	Superoxide dismutase	25.9	2.4	2.2

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Table 6.2 – *Continued from previous page*

Protein ID	Chifu homologue	Protein name	Score	Log_2 FC	-log P-value
A0A3P5YUS8	Bra031642	Superoxide dismutase	20.5	1.6	2.4
UTP/CTP biosynthetic process					
A0A397YZT6	Bra038621	Nucleoside diphosphate kinase	18.1	1.2	1.2
A0A397YEX8	Bra030844	Nucleoside diphosphate kinase	62.3	1.1	2.1
AA metabolic process					
A0A3P6A7G2	Bra023450	3-isopropylmalate dehydrogenase	4.7	2.5	1.3
A0A3P6B037	Bra026621	Glutamate decarboxylase	119.00	-3.8	2.6
A0A3P5Z1R4	Bra011449	Phospho-2-dehydro-3-deoxyheptonate aldolase	56.00	-3.8	2.7
A0A3P6CBN9	Bra037682	Cysteine synthase	17.07	-3.2	1.2
A0A3P5ZPF9	Bra005864	Anthranilate synthase	7.56	-1.8	1.4
A0A3P6A7T9	Bra023651	Uncharacterized protein	5.43	-1.6	1.4
A0A3P6ADR9	Bra013209	Indole-3-glycerol-phosphate synthase	8.81	-1.6	1.9
Misc					
A0A3P6A432	Bra037029	UDP-sulfoquinovose synthase1 *	20.8	4.10	1.9
A0A397Y6V5	Bra030945	PEPC 1	64.3	1.3	2.0
A0A3P6CNL0	Bra032681	Carboxypeptidase	2.8	2.9	3.8
A0A3P5Z0Z4	Bra011792	PEPCK (ATP) 1	8.0	-1.4	2.7
A0A3P6CTA5	Bra004710	PEPC 2	2.4	-1.1	1.5

To see if there was also an increase in the APase activity to correlate with the increased abundance of PAP proteins detected in response to Pi starvation, an in gel APase activity assay was performed on the shoot proteins, APase activity is indicated by red coloration (Fig. 6.8). There was increased APase activity in Pi starved shoot proteins compared to the control proteins, with clear coloration at approximately 130 KDa and 30 KDa, while there was no obvious coloration detected in the control samples. Showing that there is also an increase in activity of APase proteins in response to Pi starvation. Indicating that the change in the proteome of *B. rapa* shoots in response to Pi starvation also leads to a change in protein activity levels, the changes could be part of a PSR to better enable the plants to adapt to deficient Pi conditions and improve their Pi efficiency.

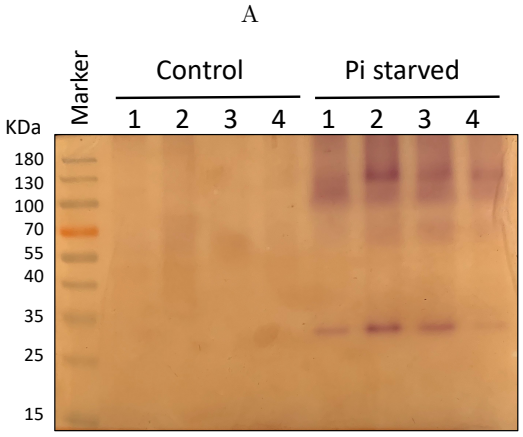

Figure 6.8: *B. rapa* shoot phosphatase activity in response to Pi starvation: In gel APase activity assay on protein extracts from *B. rapa* shoots. 2.5 ug of protein separated in native PAGE gel, and incubated in APse activity buffer containing PNP and fast black K, where APase activity is indicated by red coloration.

6.2.3 PSR in *B. rapa* shoots results in an altered phosphoproteome indicating a change in signalling events

The shoots of *B. rapa* have already displayed a significant transcriptomic and proteomic response to phosphate starvation. Next I will look at the shoots phosphoproteomic response to Pi stress, to identify any changes that could potentially be involved in signalling events during a PSR. Phosphopeptide enrichment was performed on protein extracts of *B. rapa* shoots after a PSR was initiated using TiO_2, the phosphopeptides were then identified using MS.

After the MS analysis a total of 61,910 spectra were recorded and assigned to 1175 different phosphopeptides with an average phosphopeptide enrichment efficiency of 43%. After filtering of potential contaminates, low confidence peptides, and a localisation score of at least 0.75, and further filtered so that the phosphopeptide had to be identified in all three biological replicates in a Pi condition. After filtering 462 phosphopeptides were identified which were assigned to 360 different proteins.

Using the LFQ intensity values the identified phosphopeptides were quantitatively analysed to determine changes in the phosphoproteome in response to Pi starvation. A PCA was performed and shows a clear separation between the different Pi conditions, with a component 1 variance of 60.6% (Fig. 6.9A). Indicating a difference in the phosphoproteome of *B. rapa* shoots in response to Pi starvation.

A standard two sample t-test was performed on the phosphopeptides LFQ intensity values to identify phosphopeptides that are significantly changed in abundance in

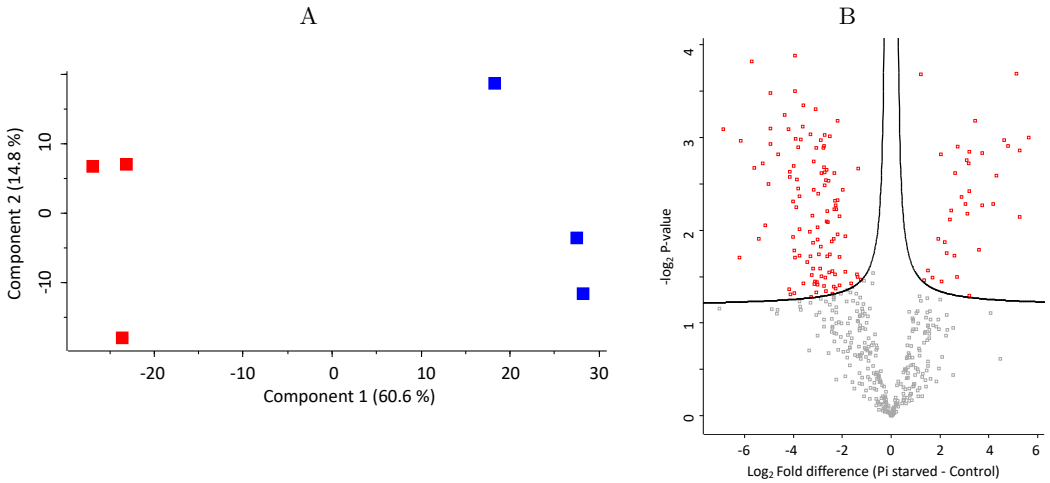

Figure 6.9: Quantitative analysis of *B. rapa* phosphoproteome in response to Pi starvation: The phosphopeptides identified in the phosphoenriched shoot samples were analysed in Perseus using their LFQ intensity values. A) PCA plot of phosphopeptides, Pi starved samples indicated in red, control samples indicated in blue. B) Volcano plot of phosphopeptides, using a student two sample t-test to identify phosphopeptides significantly changed in abundance, $\log_2$ fold change is plotted against -log P-value. Red points show the 135 phosphopeptides which are significantly changed in abundance in response to Pi starvation, FDR:0.05 s0:0.1.

response to Pi starvation, using a FDR:0.05 and s0:0.1. A volcano plot was used to represent this data, plotting the $\log_2$ FC difference, Pi starved intensities minus the control intensities, against the -log p-value, points in red represent the phosphopeptides significantly changed in abundance (Fig. 6.9B). Of the 462 phosphopeptides identified 135 were determined to be significantly changed in abundance, 34 were more abundant in response to Pi starvation while 101 were less abundant.

The majority of the phospho modifications identified on the phosphopeptides are on serine residues at 88%, 6% are phosphotyrosine, and 6% are phosphothreonine. The majority of the peptides detected are mono phosphorylated at 91%, 6% have 2 phospho modification then only 3% have three phospho modifications.

GO-term enrichment analysis was used to see if there were any common functions or processes in the phosphorylated proteins which changed in abundance in response to Pi starvation Phosphopeptides that increased in abundance were enriched in a few biological processes; Sucrose biosynthetic process, tricarboxylic acid cycle, and mRNA splicing. Molecular functions that were enriched included sucrose-P synthase activity and PEPC activity. The phosphopeptides that decreased in abundance in response to Pi starvation were associated with proteins that had an enrichment in regulation of translation, and mRNA binding.

A subset of the phosphopeptides detected to change in abundance are shown in Table 6.3, the peptides displayed are grouped based on their protein groups and by functions/processes which were enriched in response to Pi starvation. Not all the significantly changed peptides are shown to make the table more manageable, so only phosphopeptides of interest and with a $\log_2$ fold change of at least 1 or are part of protein groups or catagories enriched in relation to Pi starvation.

Table 6.3: *B. rapa* shoot phosphopeptides that changed in response to Pi starvation: A subset of the phosphopeptides which changed in abundance in response to Pi starvation grouped by processes and protein groups. Protein ID's and names from uniprot. The *B. rapa* chifu homologue from BLAST. Site is the position within the proteins phosphorylated. $\log_2$ fold change, -log P value from the calculated FC by students two sample t-test. * Indicates proteins that were uncharacterised so are named using their closest *Arabidopsis* homologue.

Protein ID	Chifu homologue	Protein name	Sequence window	$\log_2$ FC	-log P-value	Site
Phosphatase						
A0A3P6BYS0	Bra039808	Inorganic diphosphatase	ERIL**S**SLTHR	5.63	2.99	S29
A0A3P5YLB1	Bra031626	Serine/threonine-protein phosphatase	RQL**S**IDQFE	-4.0	1.8	S602
Phosphoenolpyruvate carboxylase						
A0A3P5Z9C2	Bra027293	PEPC 3	IEKMA**S**IDAQL	3.73	2.83	S11
A0A3P5YR48	Bra004710	PEPC 2	LKKMA**S**IDAQL	3.73	2.83	S11
A0A3P6CTA5	Bra004710	PEPC 2	LKKMG**S**IDAQL	2.87	2.36	S11
A0A397Y6V5	Bra030945	PEPC 1	LEKMA**S**MDVH	5.27	2.14	S11
sucrose biosynthetic process						
A0A397XKW7	Bra002289	Sucrose-phosphate synthase 1	DIQDI**S**LNLRF	4.31	2.59	S696
A0A3P6A8T1	Bra033195	Sucrose-phosphate synthase 4	EPISD**S**LGDVD	1.22	3.68	S714
A0A3P6AP98	Bra020096	Sucrose-phosphate synthase 1	SFRDI**S**LNLRF	5.12	3.69	S699
GTPase activity						
A0A3P5ZXN0	Bra000005	Arf-GAP domain-containing protein	VGFGS**S**PGPAP	-2.68	2.54	S235
A0A3P5YZQ6	Bra019950	Dynamin GTPase	NGDLP**S**PGSGS	-4.17	1.37	S913
A0A3P5ZVR2	Bra035433	Dynamin GTPase	MRQSF**S**EGSLD	-4.03	1.93	S743
A0A3P5ZI83	Bra040376	Ras-related protein RABA5b*	YKAQL**S**VNRVS	-2.89	1.82	S192
Kinase						
A0A3P5ZS05	Bra006812	Protein kinase domain-containing protein	QQYPD**S**PRFN	2.61	2.62	S178

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Table 6.3 – *Continued from previous page*

Protein ID	Chifu homologue	Protein name	Sequence window	log_2 FC	-log P-value	Site
A0A398AUQ0	Bra040509	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase	LTRQG**S**GIIGT	-5.3	2.7	S363
A0A3P5Y6T9	Bra007738	Protein kinase domain-containing protein	VGSR**S**AGGG	-3.0	1.7	S27
Transporter						
A0A3P5ZQL2	Bra011109	Regulator of Vps4 activity in the MVB pathway protein*	LSYGM**S**PPRE	3.11	2.18	S660
A0A3P5YH87	Bra019446	SEC7 domain-containing protein	SAAEV**S**VPSS	-3.9	3.0	S1506
A0A3P5Y982	Bra026864	PHO1 homologue *	LYRTF**S**GLVST	-3.2	1.4	S67
A0A3P6ALK1	Bra023352	Probable magnesium transporter	DFRSY**S**ASYA	-3.2	1.9	S33
A0A3P5YXQ5	Bra026156	ABC transporter G *	LEDIF**S**PSAR	-2.7	3.0	S35
A0A3P6CR55	Bra015484	GAT domain-containing protein	ELGL**S**SDED	-1.4	1.5	S324
Misc						
A0A3P5Y4E4	Bra040854	Patellin-2 *	IAHTE**S**FKEEG	5.25	2.86	S96
A0A3P6ADU5	Bra034020	Midasin	LAREG**YY**LLAE	4.78	2.91	Y1283
A0A3P5YHS7	Bra029309	Ribonuclease	YGDIQ**S**DDEDS	-3.2	2.4	S969
A0A397YDI3	Bra016282	Eukaryotic translation initiation factor 5A (eIF-5A)	M**S**DEEH	-6.9	3.1	S2
A0A397YFU7	Bra030651	Glyco_transf_20 domain-containing protein	MISR**S**YTNL	-5.2	2.1	S5
mRNA Splicing activity						
A0A3P5YYP5	Bra026037	RRM domain-containing protein	RRLPD**S**PPRR	4.18	2.28	S240
A0A397ZHP8	Bra014741	RRM domain-containing protein	SRRSY**S**PGYE	3.73	2.26	S225
A0A3P5Z3D4	Bra024276	RRM domain-containing protein	PR**S**ASPLKA**S**P	3.19	2.84	S259, S266
A0A3P5YR39	Bra026712	RRM domain-containing protein	RRLPV**S**SPRR	3.17	2.72	S240
A0A3P5Z4U3	Bra013895	RS40*	KRERT**S**PDYG	2.45	2.22	S262

Common motifs that are enriched within phosphopeptides can give an idea about what kinases commonly act on the phosphopeptides identified, hence give an indication of the phosphoylation events that occure in response to different conditions. Motif analysis using MoMo motif-x was used to identify enriched motifs with a Fishers exact test with a P-value threshold of <1.0e-6, using *B. rapa* background reference [Cheng et al., 2019]. Motif analysis on all phosphopeptides identified in the shoot phosphoproteome revealed that there were 9 motifs that were enriched (Table 6.4). Of the motifs identified only the -SP- motif was enriched in the phosphopeptides that were identified to both increase and decrease in response to Pi starvation, indicating a simularity in the kinases that may be involved in their phosphorylation. -SP- motifs are targets of MAPK, SnRK2, RLK, AGC kinase, cyclin-dependent kinases, calcium-dependent protein kinases, and STE20-like kinase [van Wijk et al., 2014].

Analysis of the phosphoproteome displays a significant change in response to Pi starvation in the shoots of *B. rapa* plants. Suggesting that signalling events that occur change during Pi deficient conditions. These signalling events could be involved in different PSR signalling pathways that enable the plants to improve their Pi efficiency.

Table 6.4: Motif analysis of *B. rapa* shoot phosphoproteome: Phosphopeptides detected in *B. rapa* shoots analysed using MoMo motif-x to determine enriched motifs, with a fishers exact test with a P-value threshold of <1.0e-6, using *B. rapa* background reference [Cheng et al., 2019]. Arrows indicate Motifs that are enriched in the phosphopeptides identified to either increse or decrease in abundance in response to Pi starvation.

Motif	Foreground matches	Background matches	Score	Significantly changed phosphopeptides
.....SP.....	217	68934	34.23	↓ ↑
...R..S.....	76	69491	19.71	
....P.SP.....	54	5633	171.66	
.....SP.R...	38	3755	96.71	
.....SPK....	37	3359	120.1	
.....S.D....	36	65343	7.47	
.....SPR....	33	4118	72.85	
...R..SP.....	27	3904	53.89	
.....TP.....	10	37592	6.26	

6.3 Discussion

The sensitivity of RNA-seq analysis has allowed the identification of transcripts which we do not see in the proteomics data. There are multiple reasons this could be the case, the level of high abundant proteins can mask the presence of low abundant proteins hence some get missed. There is also the chance that some proteins do not digest into peptides that ionise well therefore is difficult for the MS to identify in a complex mixture, these are problems that RNA-seq is not inhibited by. But there is always the risk that just because the expression of the transcripts is increased does not always mean there is more of the finished protein product prescent, so what we are seeing is not necessarily accurate at the protein level within the plants.

Similarities between the transcriptomic data and the proteomics data show that for both there is an increased expression and abundance of PAPs. Three PAP genes were in the top 20 genes that most increased in expression during Pi starvation, these PAPS were BrPAP 6, 17, and 5, another nine PAP genes were also significantly increased in expression during Pi starvation. There was also two PAP proteins (BrPAP1 and BrPAP15) that were significantly increased in abundance which corresponded to two of the PAP genes that increased in expression. There were various other phosphatase proteins which increased in abundance, pyrophosphatase proteins and inositol phosphatase proteins. There was also an enrichment for the GO term acid phosphatase activity, in both the transcriptomic and proteomic datasets indicating the increase in abundance was to a significant ammount. To match with the increased abundance there was also increased APase activity in the protein extracts from Pi starved shoots, shown in the in-gel APase activity assay (Fig. 6.8). The phosphatases increased in abundance could function to improve Pi efficiency by remobilising Pi from storage sources, inositol phosphate is a common form of Pi storage in vacuoles during Pi sufficient conditions [Yang et al., 2017], this can then be utilised during Pi starvation and the Pi released by inositol phosphatases to be utilised, improving the plants Pi access during Pi shortage. The remobilisation of Pi from storage helps to maintain intracellular homeostasis [Fang et al., 2009]. The SPX domain containing transmembrane transporter (Bra034426) identified to increase in expression during Pi starvation is a homologue of a vacuole phosphate transporter identified in both rice and *Arabidopsis*, but its role in Pi distribition is unclear as in *Arabidopsis* in appears to function as an influx transporter so functions to store Pi in vacuoles [Liu et al., 2016], while the rice homologue has Pi efflux capabilities, therefore can distribute Pi from strorage during Pi stress [Liu et al., 2016, Wang et al., 2015]. This could potentailly indicate a transporter involved in

the remobilization of Pi during Pi starvation.

There were numerous other transporters identified to have increased expression durinf Pi starvation. PHO1 genes expression is significantly increased in response to Pi starvation, they have been reported to shuttle Pi into the xylum for distribution to the rest of the plant in *Arabidopsis* [López-Arredondo et al., 2014]. PHT1;5, a plasma membrane Pi transporter was also seen to increase in expression during Pi starvation and is also implicated in the redistribution of Pi during Pi stress conditions in *Arabidopsis* [Nagarajan et al., 2011]. The induction of these transporters in *B. rapa* could allow the distribution of Pi from storage tissues to other areas of the plant more in need to Pi during Pi deficient conditions allowing improved Pi efficiency.

The transcriptomic data showed a variety of SPX domain containing proteins with increased expression. SPX protiens are suspected to be involved in signalling and sensing of Pi levels, but none of these proteins were identified in the shoot proteomics data. An mentioned in the introduction, SPX proteins are thought to be involved in sensing of Pi status in cells and the signalling and activation of phosphate specific transcription factors, PHR1 and PHL1, which induce the expression of PSI genes. The increased expression of the SPX domain proteins could implicate a role in Pi signalling, but at the same time proteins involved in the sensing of Pi status would not be expected to change in abundance as they would need to be present regardless of Pi levels in order to sense it, so it is the downstream effects of the signalling pathways that are expected to change in response to Pi starvation.

The flavonoid biosynthesis pathway was a significantly enriched GO term processes identified in the genes that incresed in expression during Pi starvation. Flavonoid biosynthesis is involved in the production of anthocyanin; the pigmentation that causes plant leaves and shoots to darken green [Dao et al., 2011], and is a known PSR [Marschner, 2011]. Three of the genes involved in flavonoid biosynthesis whose expression levels increased during Pi starvation were three of the top 20 most increased in expression; Chalcone synthase 1 and 3, and Flavonoid 3'-monooxygenase, indicating a strong induction of this PSR.

The expression levels of various genes involved in lipid remodeling were increased during Pi starvation. Monogalactosyldiacylglycerol (MGDG) synthase 2/3 genes were two of the genes most increased in expression during Pi starvation. MGDG is a non phosphorus containing glycolipid which can be used to replace membrane phospholipids during Pi deficiency, when Pi from phopholipids gets recycled and re-

purposed. MGDG synthesis occurs in the plastid envelope by MGDG synthase. The AtMGDG2 and 3 in *Arabidopiss* genes have shown an increase in expression during low Pi conditions [Basnet et al., 2019, Kobayashi et al., 2009] and are therefore a part of the lipid remodelling PSR, along with GDPD and phosphoplipase genes which encode for proteins involved in the breakdown of phospholipids [Ramaiah et al., 2011].

Phosphoproteomic changes in response to Pi starvtion could help to identify the Pi related signalling within the shoot material. Sometimes PTM are required to change the activity of the gene products and can cause signalling cascades in response to stimuli such as stress conditions. Therefore looking at the phosphoproteomes to see differences in phosphorylation could give an indication in the changes in signalling or activity of the proteins present.

There was increased abundance of three sucrose-P synthase (SPS) proteins (two BrSPS1 proteins, and one BrSPS4) identified in the phosphoproteome to have increased phosphorylation in response to Pi starvation. SPS proteins activity is regulated by its phosphorylation status [Toroser and Huber, 1997], and they play a role in sucrose biosynthesis. Sucrose plays a role during signalling and is involved in shoot to root communicaion during Pi stress. It has been shown previously that decreasing Pi levels leads to increased sucrose within plant shoots [Hammond and White, 2008], and has been documented that an increase in sucrose is correlated with increased ethylene biosynthesis and signalling which acts to induce PSI gene expression [Song and Liu, 2015]. Hence showing sucrose signalling could be linked with ethylene signalling, which is a component of phosphate response signalling, and also acts as a negative regulator for the anthocyanin accumulation that occurs during Pi deficiency [Song and Liu, 2015, Wang et al., 2012]. There was an increase in abundance of one of the BrSPS1 proteins (A0A3P6AP98) also identified in the shoot proteome as well as the phosphoproteome during Pi starvation, which could indicate an overall increase in abundance of this protein rather than an increase in its phosphorylation. AtSPS1, AtSPS2 and AtSPS4 phosphopeptides were also found to increase in abundance in *Arabidopsis* seedlings phosphoproteome in response to Pi starvation in chapter 3, with the phosphorylation site in AtSPS4 and BrSPS4 identified to be at the same coresponding residues in both *B. rapa* and *Arabidopsis* identified at S714 and S717 respectively. Indicating there could be a simular PSR between the species ragarding sucrose synthesis, but the specific functions of these proteins and the function the phosphorylation has at these residues would need further research to determine their roles in a PSR.

Sucrose supply is also partly involved in regulating of the PTM of PEPC proteins [Plaxton and Shane, 2015]. PEPC phosphopeptides were identified in the shoot phosphoproteome to increase in abundance in response to Pi starvation, 4 PEPC proteins (BrPEPC1, two BrPEPC2, and BrPEPC3) with phosphorylation on their S11 residue increased in abundance. However while the shoot proteome also shows an increased abundance of BrPEPC1, it also showed that BrPEPC2 (A0A3P6CTA5) decreased in abundance during Pi starvation despite the increase in phosphorylation detected, indicating that there was increased phosphorylation opposed to an overall increase in protein abundance, but I can not confirm this exactly. There is also a decrease in abundance of a BrPEPCK 1 (A0A3P5Z0Z4) protein in the shoot proteome, while shoot transcriptome had an increased expression of a different homologue of the BrPEPCK 1 gene (A0A397YFT7) and decreased gene expression of BrPEPCK2, which indicates that the expression and abundance of different PEPC and PEPCK proteins and their phophorylation status does not always correlate as PEPC is a phosphoylation target of PEPCK [Chen et al., 2008].

6.4 Conclusion

The aim of this chapter was to identify how *B. rapa* shoots respond to Pi starvation, and to identify potential targets that could be involved in improving the plants Pi efficiency as a part of their PSR. I was able to conlcude that transcriptome, proteome and phosphoproteome all changed significantly during Pi starvation, some of these changes are consistently identified through the different analytic techniques, while other changes were only observed by a specific analytical technique. A common change was that members of the PEPC family were identified in the transcriptome, proteome and phosphoproteome to various levels, indicating a role in PSR in *B. rapa* shoots. Another common occurance that was consistently seen was an increased expression and abundance of phosphatase proteins, and proteins involved in carbohydrate metabolic process. Some of the responses observed could be implicated in improving the Pi efficiency of *B. rapa* by improving the Pi utilisation efficiency. Such as through the release Pi from Po sources by phosphatase proteins, and by releasing stored Pi for redistribution and repurposing. The changes in phosphopeptides identified could indicate signalling players that could be involved in signalling pathways during a PSR to help the plants further signal the need, and the pathways for improved Pi efficiency. However, the specific functions and roles of the identified targets that change during Pi starvation will need further research to answer how they function to improve Pi efficiency, and further work to be able to utilise them

for further improvements in Pi efficiency during Pi deficient conditions.

Chapter 7

General Discussion

7.1 Comparative analysis of *B. rapa* and *Arabidopsis* phosphate responsive proteomes

craig made a test.

The aims of the work carried out in this thesis were to identify the changes in the proteome of *B. rapa* plants in response to Pi deficiency, and to identify those proteins and protein groups which may play a role in the improvement of phosphate utilisation or uptake efficiency in response to Pi starvation. This was done by identifying the changing proteins in *B. rapa* exudates, roots and shoots in response to Pi starvation, as well as identifying and comparing to *Arabidopsis* proteomic changes. Proteins which commonly responded to Pi starvation were PAP proteins, inositol phosphatases, RNases, and proteins involved in lipid remodelling and sucrose metabolism. The changes in these proteins could be the key to further understanding Pi efficiency responses in *B. rapa*.

The comparison of *B. rapa* proteins to the *Arabidopsis* proteins identified to change in response to Pi stress demonstrate how different the crop species is to the model organism in their Pi starvation responses. The improved characterisation of *Arabidopsis* helps to assign functions to some of the uncharacterised *B. rapa* proteins identified, if we assume that the *B. rapa* protein ortholog shared by *Arabidopsis* functions and behaves in similar ways, but further functional experimentation will be needed to confirm this, which will be discussed in the future work section.

Increased root secretion of proteins in response to Pi starvation is a common PSR

displayed by plants. In this study both *Arabidopsis* and *B. rapa* displayed a significant change in their exudate proteome in response to Pi starvation, and I wanted to determine how similar the responses were between the two species. Proteins from *B. rapa* R500 exudates and the *Arabidopsis* Col-0 exudates which change in response to Pi starvation were compared to determine if they are conserved or if they are specific. The *Arabidopsis* homologues of the *B. rapa* exudate proteins were used to compare with the Col-0 exudate proteins identified to determine similarities as they are likely to share similar functions. The proteins which changed in abundance in response to Pi starvation in both the *B. rapa* and *Arabidopsis* exudate proteomes are displayed in Table 7.1. Only 11 out of the 195 *B. rapa* exudate proteins identified to respond to Pi starvation were also identified in *Arabidopsis* to respond to Pi, indicating only a 5.6% homology to the *Arabidopsis* exudate proteomic response.

There were seven proteins identified to increase in abundance in both the *Arabidopsis* and *B. rapa* exudates in response to Pi starvation, these consisted of two GDPD proteins and a ribonuclease (T2) 1 protein which are known to be Pi responsive, as well as a germin-like protein, auxin-induced in root cultures protein 12, HIPL1 protein, and peroxidase 52. Only one protein decreased in abundance in both the *Arabidopsis* and *B. rapa* exudates; a peptidyl-prolyl cis-trans isomerase CYP18-4, which aids protein folding. There were three proteins which displayed opposite responses, increasing in *B. rapa* and decreasing in *Arabidopsis* exudates in response to Pi starvation; translational-controlled tumor protein 1, triosephosphate isomerase, and an elongation factor, none of which appear to have a specific Pi response function.

Table 7.1: Comparison of *Arabidopsis* Col-0 and *B. rapa* R500 exudate proteome in response to Pi starvation: The proteins identified to significantly change in abundance in response to Pi starvation in both *Arabidopsis* Col-0 and *B. rapa* R500. *Arabidopsis* homologues of the *B. rapa* exudate proteins were used to compare with the Col-0 exudate proteins. Arrows indicate whether they increased or decreased in abundance in the exudates in response to Pi starvation.

Arabidopsis Protein ID	*B. rapa* Protein ID	Protein Name	Col-0	R500
At3g16640	A0A398A1Q3	Translationally-controlled tumor protein 1	↓	↑
AT5g05340	A0A3P6CR30	Peroxidase 52	↑	↑
At2g02990	A0A3P6AUK7	Ribonuclease 1	↑	↑
At1g74790	A0A3P6BY14	HIPL1 protein	↑	↑
At3g07390	A0A398A6H9	Auxin-induced in root cultures protein 12	↑	↑
At4g14630	A0A3P5XY25	Germin-like protein subfamily 1	↑	↑
At4g26690	A0A3P6A2W2, A0A3P5ZTK7	GDPDL3	↑	↑
At5g55480	A0A3P6CG48	GDPDL4	↑	↑
At4g34870	A0A3P5ZP47	Peptidyl-prolyl cis-trans isomerase	↓	↓
At3g55440	A0A3P6CCR3	Triosephosphate isomerase	↓	↑
At1g09640	A0A3P5YUY3	Probable elongation factor 1-gamma 1	↓	↑

PTM are often are part of signalling pathways in response to stress conditions, changes in the phosphoproteome during Pi starvation could indicate proteins and phosphorylation events that are involved in Pi stress signalling pathways, and may allow some insight into how the plants respond to the environmental cues. The comparison for the *B. rapa* and Col-0 phosphopeptides are not completely comparable, as the Col-0 phosphoproteomics looking at Pi starvation responses were on whole seedlings, while the *B. rapa* phosphoproteomics were done separately on the root and shoot material. The proteins detected which did have significant changes in phosphopeptides identified are displayed in Table 7.2 and also display the phosphorylation sites identified which increased or decreased in response to Pi starvation. Of the 281 phosphorylated proteins identified in *B. rapa* phosphoproteome to respond to Pi starvation only 27 (10%) were also identified in the *Arabidopsis* phosphoproteome to also respond to Pi starvation, but they did not always respond in the same way or have the same phosphorylation site.

The common proteins which displayed an increase in phosphorylation in response to Pi starvation in both Col-0 and R500 plants included, PEPC1 and PEPC3, SPS1 and SPS4, a soluble inorganic pyrophosphatase, annexin protein and a protein kinase. These proteins could be involved in improving Pi responsive signalling between both plant species. The PEPC proteins and protein kinase are phosphorylated on the same relative residues, while the SPS and annexin proteins are phosphorylated on different relative residues in the *B. rapa* and *Arabidopsis* homologues.

Table 7.2: Comparison of *Arabidopsis* and *B. rapa* phosphoproteome: The proteins which had significantly changed phosphopeptides identified in response to Pi starvation in both *Arabidopsis* Col-0 and *B. rapa* R500. *Arabidopsis* homologues of the *B. rapa* proteins were used to compare the phosphoproteomes. Arrows indicate whether the identified phosphomodification increased or decreased in abundance in response to Pi starvation.

Arabidopsis Protein ID	*B. rapa* Protein ID	Protein Name	Col-0	R500 Shoot	R500 Root
AT3G14940	A0A3P5Z9C2	PEPC 3	↑ S11	↑ S11	↑S11
AT1G53310	A0A397Y6V5	PEPC 1	↑ S11	↑ S11	
AT3G53620	A0A3P6BYS0	Soluble inorganic pyrophosphatase 4	↑ S28	↑ 29S	
AT4G10120	A0A3P6A8T1	Probable sucrose-phosphate synthase4	↑ S717,148,180	↑ S714	
AT5G20280	A0A3P6AP98, A0A397XKW7	Sucrose-phosphate synthase 1	↑S688,S700,S152	↑S696,699	
AT1G35720	A0A3P5ZFG8	Annexin D1	↑ S70	↑S14	
AT5G57610	A0A3P5ZS05	Kinase superfamily with octicosapeptide	↑ S169,945,220	↑S178	
AT3G55460	A0A397ZHP8	Serine/arginine-rich SC35-like splicing factor SCL30	↑ S182	↑S225	↓S6,S11

Continued on next page

Table 7.2 – *Continued from previous page*

Arabidopsis Protein ID	*B. rapa* Protein ID	Protein Name	Col-0	R500 Shoot	R500 Root
AT5G15680	A0A3P6A6Z3	ARM repeat superfamily protein	↑S1075		↓1906
AT4G13350	A0A3P6BZE9	Nuclear shuttle protein-interacting GTPase	↑ S219		↓S407, 150
AT3G13530	A0A3P6A9F4	MAP3K epsilon protein kinase 1	↑ S1005,1013		↓S386
AT3G05090	A0A3P5ZX56	Transducin	↑S369		↓S381,S191
AT4G31880	A0A3P6AI91	Transcriptional regulator	↑ S557	↓S555	
AT1G59610	A0A3P5ZVR2	Dynamin-2B	↓ S719	↓S743	↓S743
AT2G15860	A0A3P6A033 A0A3P6AQ02	BAT2 domain protein	↓S190	↑S27	↓S181
AT5G03280	A0A3P6CR21	Ethylene-insensitive protein 2	↓S655		↓S651
AT5G25270	A0A3P5Z1Y7	Ubiquitin-like superfamily protein	↓S632		↓S616
AT1G80350	A0A3P6BUJ1	Katanin p60 ATPase-containing subunit A1	↓S92		↓S96
AT2G20190	A0A3P5XWH3	CLIP-associated protein	↓S612		↓S303,320,Y319
AT5G63190	A0A3P5Z0I8	MA3 domain-containing protein 6	↓S29		↓S81
AT1G74910	A0A3P6BNJ9	ADP-glucose pyrophosphorylase	↓S218		↓S218
AT4G38550	A0A3P6C5S1	Phospholipase-like protein	↓S32		↓S292,S301,S201
AT4G15545	A0A3P5ZJ65	Uncharacterized protein	↓S217	↓S2,S215	
AT5G18230	A0A3P5ZFM2	Transcription regulator NOT2	↓ S457	↓S482,S434	
AT1G24706	A0A3P6BZW5	THO complex subunit 2	↓S1458	↓S1652	
AT1G01320	A0A3P6D221	Tetratricopeptide repeat (TPR)-like superfamily protein	↓S149	↓S149	
AT1G31870	A0A3P5Z780	Pre-mRNA-splicing factor of RES	↓S286, S337	↑S250	

The root and shoots of *B. rapa* have also displayed vastly different phosphoproteomes with only a few similarities identified in the phosphopeptides which changed in abundance in response to Pi starvation, indicating different signalling events in the tissues during Pi starvation which could lead to the different Pi efficiency responses. The *B. rapa* root phosphoproteome only had a total of 6 phosphopeptides and proteins increased in abundance in response to Pi starvation, and two of them

were identified to also increase in abundance in *B. rapa* shoots; BrPEPC2 and BrPEPC3 phosphorylated at S11. *Arabidopsis* also had the AtPEPC3 phosphorylated at S11 identified to increase in abundance in response to in Pi starvation. The AtPEPC3 had previously been documented to being phosphorylated in PhosPhAt, but the *B. rapa* proteins had not previously been reported to have this phosphoserine modifications. The presence of these PEPC proteins between species and tissue types indicates its role in the plants PSR is conserved.

Even though not many of the same specific protein homologues are observed in both *B. rapa* and *Arabidopsis* to respond to Pi starvation, there are a few different proteins that do belong to the same protein family which respond in the same way. For example PAP and pyrophosphatase proteins are increased in abundance in the exudate proteomes of both the Col-0 and R500 plants starved of Pi. These results could indicate that while some of the phosphatase responses to Pi starvation are similar as a group between the species, the specific proteins are less conserved in their Pi starvation response therefore respond differently, this would not be surprising as they are from different species so would expect some differences. The same could be said for the potential signalling pathways, as there were only a few phosphorylated proteins that increased in abundance in response to Pi starvation identified in both *B. rapa* and *Arabidopsis*, and even less which had the same modified residues, indicating different responses to Pi stress between the different species. The differences identified may also be due to the slightly different growth conditions and the age of the plants. The *B. rapa* plants were 31 days old while the *Arabidopsis* plants were ten days old when they were harvested, so they may have different responses based on their different ages, therefore not completely comparable. The *Arabidopsis* plants were also starved of Pi for five days while *B. rapa* was starved for eleven days, therefore the differences in identified proteins and phosphorylation could be due to a difference in longer term responses to Pi starvation. But seeing what proteins are similar is still useful to see what proteins are consistently changed despite the differences in plant and growth system to identify conserved responses to Pi deficiency in different plant species, as these could offer the most broad targets for improving Pi efficiency.

The low similarity in proteins identified between *B. rapa* and *Arabidopsis* means studying *B. rapa* is valuable as we can not predict the crop species response to Pi starvation based from the model organisms response. As the specific proteins which do respond may not be the same or be close homologues to each other even if the general protein family does have a conserved PSR.

7.2 The changes in PAP proteins in response to phosphate starvation and how PAPs could contribute to improved P efficiency in plants

There have been a few protein groups which have consistently been observed in samples to respond to Pi starvation, such as the Purple Acid Phosphatase proteins, which are a key PSR. These were observed to be secreted from both *Arabidopsis* and *B. rapa* roots. PAPs were also detected within the membrane associated proteome of *B. rapa* roots, the soluble root proteome, and the soluble shoot proteome, with their abundance increasing significantly during Pi starvation. PAP transcripts were also detected to have significant changes in expression in response to Pi stress within *B. rapa* shoots. APase activity assays have also revealed an increased APase activity in roots and shoots of *B. rapa* as well as on the roots of *Arabidopsis* during Pi starvation. Unfortunately it has not possible to identify which phosphatases are responsible for the increased activity, but the increased APase activity and increased abundance of PAP proteins correlates nicely.

Arabidopsis has 29 PAP proteins classified as AtPAP1 to AtPAP29 [Li et al., 2002]. While the *B. rapa* genome has 83 potential phosphatase protein coding genes, of these there are 37 PAP coding genes [Wang et al., 2011b, Xie and Shang, 2018], which are classified based from their *Arabidopsis* homologues and using Uniprot along with cluster alignment [Sievers and Higgins, 2018]. The increase in copy number of complete PAP genes in *B. rapa* (37) compared with *Arabidopsis* (29) is generally equal to the increase in total gene number (41,019 and 35,386) as determined by Xie and Shang [2018].

The PAP family in plants can be characterised based on whether they are high or low molecular mass proteins [Zhu et al., 2020]. The family is split into 3 main groups; 1,2 and 3 which are further split into another 8 sub groups. Of these groups, group 3 is typically considered to be low molecular mass proteins while group 1 and 2 are considered high molecular mass proteins [Li et al., 2002]. These family groups of PAPs have been classified from *Arabidopsis* but are not just found in *Arabidopsis* plants and are shared by a variety of plant species, but the nomenclature is variable, for example group 3b is classed as a separate group 4 in tea plants [Yin et al., 2019].

A phylogenetic tree displaying the relationship of the different *B. rapa* and *Arabidopsis* PAPs with each other, shows the close homology between the species and the

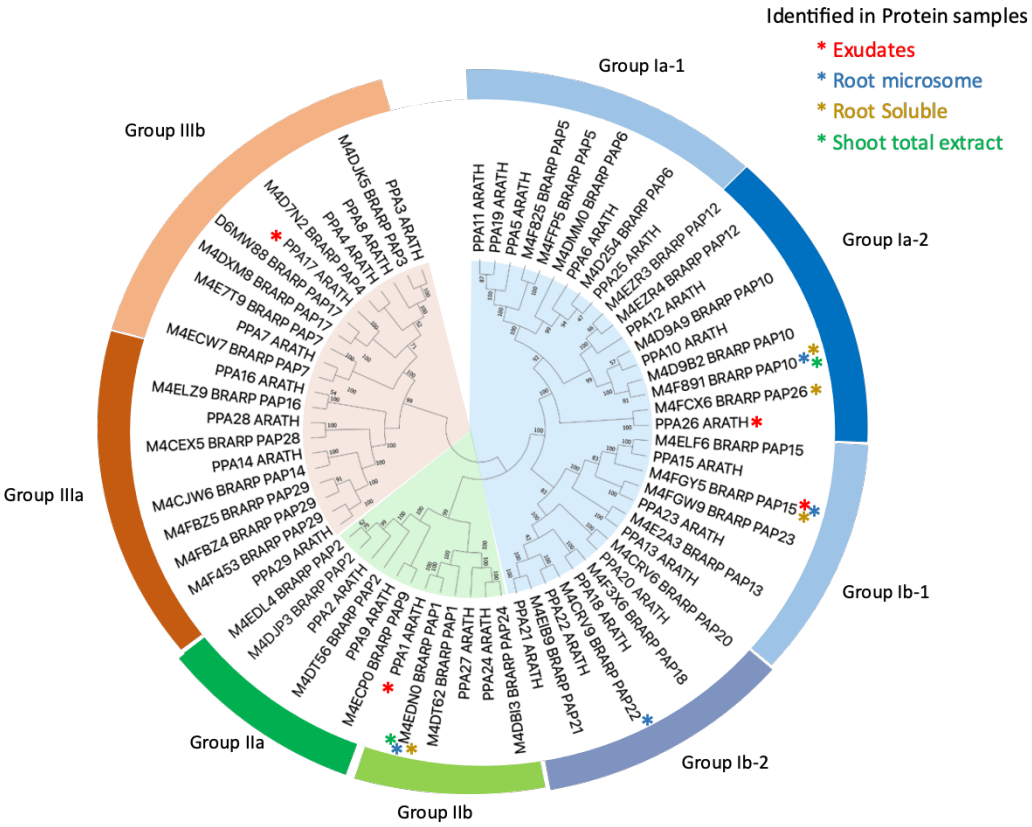

Figure 7.1: Phylogenetic tree displaying *B. rapa* and *Arabidopsis* PAPs: Clustering analysis using Clustal Omega alignment [Sievers and Higgins, 2018] of the 37 *B. rapa* PAP and 29 *Arabidopsis* PAP proteins, then Neighbor-Joining method to show the phylogenetic relationship made using MEGA5.0[Kumar et al., 2018, Stecher et al., 2020]. The PAP proteins main 3 groups are distinguished and show the split into the 8 subgroups along the outside of the tree. * Indicate if the protein was identified in my proteomic data; from root exudate samples (red *), root membrane associated samples (blue *), root soluble (yellow *), or the shoot total extracts (green *).

split of the proteins in their assigned groups (Fig. 7.1). The tree is also displaying which PAPs were identified to have an increased abundance within the different proteomes of Pi starved plants compared to control plants in my data. This shows that BrPAP1, BrPAP10 and BrPAP15 are each present in multiple proteomes, while BrPAP22 is only identified in the membrane associated proteome, and BrPAP26 only in the root soluble proteome in *B. rapa*. The phylogenetic tree shows that a variety of PAPs from groups 1 and 2 are represented in my data, while only one group 3 PAP (AtPAP17) was identified, in the Col-0 exudate samples.

I am interested in the PAPs identified within my datasets because various PAP proteins have been shown previously to play a role in improving Pi efficiency in a variety of plant species. AtPAP17 and AtPAP26 were both identified to increase in abundance in my Pi starved Col-0 exudate proteome and have both been documented previously in *Arabidopsis* to have increased expression under Pi starvation conditions and that they are functionally redundant with each other in seedlings [Farhadi et al., 2020]. AtPAP26 is reported to be secreted, and that Pi starvation conditions lead to an increase in secreted APase activity, as well as improved Pi uptake when supplied with Po sources, suggesting it has a role in Pi scavenging from rhizosphere Po sources during Pi starvation [Robinson et al., 2012, Tran et al., 2010]. AtPAP26 as been reported to be secreted alongside AtPAP12 [Robinson et al., 2012], but AtPAP12 was not detected in my data sets. AtPAP17 is also a known secreted PAP and is a duel localized protein to both plants intracellular proteome and the secretome, it has also displayed peroxidase activity but with a low affinity to hydrogen peroxide [O'Gallagher et al., 2021]. Therefore identifying the PAPs that are responsive to Pi deficiency in *B. rapa* could present candidates that could be utilised to improve *B. rapa* Pi efficiency.

AtPAP1 was identified in the *Arabidopsis* exudates, and its homologue BrPAP1 (M4EDN0), which has a 86.8% identity at the amino acid level, was identified in the *B. rapa* root membrane associated and root soluble proteomes, and in the shoot proteome with increased abundance during Pi starvation, but was not identified in the *B. rapa* exudates. AtPAP26 was also identified in the in *Arabidopsis* exudates of Pi starved plants, and its *B. rapa* homologue BrPAP26 (M4FCX6), which has a 86.5% sequence identity, was detected in the root soluble proteome but not in the exudates. These differences in localisation could be an indication that secreted PAP proteins in *Arabidopsis* are not secreted in *B. rapa* but are still responsive to Pi levels, or it could be that BrPAP1 and BrPAP26 were just not identified in the samples. Or it is possible that the differences in growth systems and time frames meant a difference in secreted proteins, with a later phosphate starvation response in *B. rapa* resulting in a difference in secreted proteins at that time while the *Arabidopsis* plants displayed an earlier Pi starvation response.

Interestingly the transcriptomic data on the shoots of Pi starved *B. rapa* identified 12 PAP genes which had increased expression in response to Pi stress, while there were 2 which had a decrease in expression levels. The two PAP proteins identified in the shoot proteome that increased in abundance during Pi deprivation (BrPAP1 and BrPAP10) were two of the PAPs identified to have increased gene expression in the

shoots. The rest of the PAP genes that increased in expression protein products were not detected using proteomics, potentially due to the increased sensitivity offered by transcriptomic analysis.

The similarities seen in PAP responses to Pi starvation in *Arabidopsis* and *B. rapa* in a variety of sample types have been interesting to see. That there is consistently an increase in PAP abundance or gene expression is encouraging to show that the species do response in a similar manner to Pi starvation. There has been consistently a significant enrichment in GO term molecular function for acid phosphatase activity in Pi starved conditions, seen in the Col-0 exudates, *B. rapa* root soluble and membrane associated proteomes, and shoot transcripts, therefore the presence of these proteins is a significant PSR that both *Arabidopsis* and *B. rapa* share. Various PAP proteins were already known to increase in abundance or expression in response to Pi starvation, with an increased activity resulting in improved Pi acquisition for the plants from Po sources. My data has identified *B. rapa* PAP proteins which are responsive to Pi deprivation, with an increased abundance of BrPAP1, BrPAP10, BrPAP15, BrPAP17, BrPAP22 and BrPAP26 observed during Pi starvation in various *B. rapa* samples. These are new protein targets that had not been documented in *B. rapa* previously to be responsive to Pi starvation and could be involved in the plants PSR to improve Pi accessibility and uptake. These PAP proteins are therefore promising candidates for further research to utilise them so that they could improve crop Pi utilisation and uptake efficiency.

7.3 Inositol phosphatases increase in abundance during phosphate starvation

Another family of proteins which are seen consistently to increase in abundance in response to Pi starvation in *B. rapa* are inositol phosphatases. Inositol-phosphate phosphatase proteins are part of the phosphodiesterase family and are a component of the inositol signalling pathways. The family is classified into 5 main members; inositol polyphosphate 5-phosphatases, suppressor of actin phosphatases, SAL1 phosphatases, inositol monophosphatase, tensin homologue deleted on chromosome 10 (PTEN)-related phosphatases [Jia et al., 2019]. These proteins function as a part of various abiotic stresses including Pi starvation. The proteins are responsible for the hydrolysis of phosphate on the inositol ring of myo-inositol (Ins), which is a component of Pi stress signalling. InsP8 presence is indicative of ample Pi availability in plant cells, with the removal of P groups from InsP8, which hence breaks it

down, signals low Pi availability which then leads to the activation the phosphate starvation induced gene expression (Fig. 1.2) [Ried et al., 2021]. Other proteins do also play a role in Ins signalling pathways during Pi stress, with bifunctional kinase proteins VIH1/2 in *Arabidopsis* also being documented to act as a phosphatases during low Pi availability to remove InsP8 levels [Zhu et al., 2019].

The *B. rapa* root and shoot soluble and root membrane associated proteomes all observed an increase of the same Inositol-phosphate phosphatase (A0A3P5YVH4) which works to release a P group from InsP. The root membrane associated proteome also observed an increased abundance in a SAC domain inositol phosphatase (A0A3P5YNG5) during Pi starvation, the *Arabidopsis* homologues of which (At-SAC7) acts to hydrolyse InsP4, and accumulates at the tips of root hairs [Thole et al., 2008]. The corresponding shoot transcript of inositol-phosphate phosphatase observed to increase in *B. rapa* shoot proteome also displayed increased expression. The shoot proteome also identified two inositol phosphatases that decreased in abundance during Pi starvation, but the corresponding transcripts did not show a significant difference in expression. Indicating various responses of different inositol phosphatases to Pi starvation.

Another group of proteins linked with InsP signalling are the inositol kinase proteins, which work opposite to the phosphatases and synthesize InsP to signal ample Pi levels. The *B. rapa* shoot phosphoproteome observed a decrease in abundance of the AtVIP1 homologue; Inositol hexakisphosphate and diphosphoinositol- pentakisphosphate kinase (A0A398AUQ0) phosphopeptide with phosphorylation at residue S363. AtVIP1 kinase functions to synthesis InsP7 and InsP8, hence signals ample Pi supply. Therefore decreased abundance or activity of VIP1 during Pi starvation correlates with decreased InsP8 to signal Pi deficiency. However the role of the phosphorylation protein at the phosphosite identified to decrease in abundance in *B. rapa* is unknown. We could speculate that it's the phosphostatus of the enzyme that is altered during Pi starvation, rather than an overall decrease in the proteins abundance, which could result in decreased activity which then has a part in InsP signalling. The kinase and phosphatase proteins that target InsP could play a role together to regulate InsP signalling during Pi starvation, but exactly how they could work together will require more research.

Despite the role of InsP and their phosphatases in Ins signalling and P storage none of the previously discussed *B. rapa* inositol-phosphatases identified to increase in abundance in response to Pi starvation have previously been linked with a PSR, nor have their *Arabidopsis* homologues. Therefore they could offer an interesting target

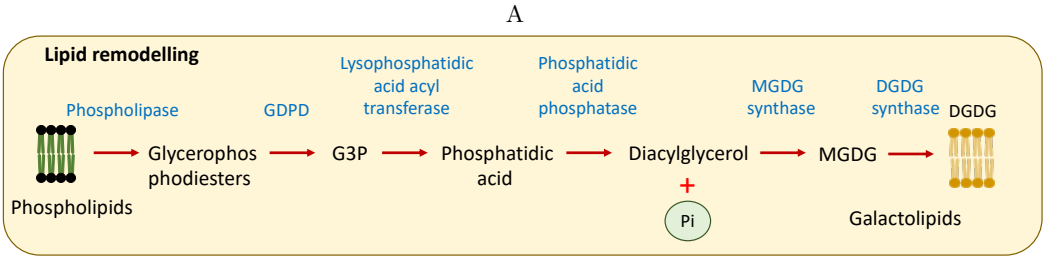

Figure 7.2: Lipid remodelling pathway: During Pi deficiency phospholipids can get broken down by a series of enzymes, displayed in blue, to be replaced with non phosphate containing glycolipids, therefore releasing a source of Pi to be utilised.

for future research to further understand Pi signalling and efficiency in *B. rapa* and in improving Pi efficiency.

7.4 Lipid remodelling as a response to phosphate deficiency

Plant membranes are a rich source of phosphate in the phospholipids which can be recycled for use during Pi limited conditions. Lipid remodelling is a PSR that multiple plant species undergo to help fuel this Pi recycling to improve plants Pi utilisation efficiency. Multiple proteins involved in this process were detected to be increased in abundance in *B. rapa* and *Arabidopsis* during Pi starvation in my protein samples.

Pi deficiency triggers the remodelling of membranes through the replacement of membrane phospholipid with non phosphorous containing glycolipids like sulfoquinovosyl diacylglycerol (SQDG), digalactosyldiacylglycerol (DGDG), and Monogalactosyldiacylglycerol (MGDG) [Tjellström et al., 2008]. This replacement of phospholipids has been observed in the roots and leaves of *Arabidopsis* with DGDG biosynthesis induced in response to Pi starvation in plastid, mitochondrial and plasma membranes [Härtel et al., 2000, Jouhet et al., 2004, Yu et al., 2002].

The lipid remodelling pathway can be summarized with the degradation of phospholipids with various enzymes, leading to the release of Pi to be repurposed, as well as the release diacylglycerol (DAG), which can then be recycled for glycolipid synthesis into MGDG then to DGDG (Fig.7.2) [Yu et al., 2021]. SQDG can also replace plasma membrane phospholipids by synthesizing UDP-sulfoquinovose then using SQD proteins to generate SQDG from UDP-sulfoquinovose and DGDG [Yu et al., 2002].

Phospholipids are broken down by phospholipase enzymes to glycerophosphodiesters, GDPD proteins then catalyze the glycerophosphodiester with water into alcohol and sn-glycerol-3-phosphate (G3P), which is a precursor for lipid biosynthesis. GDPD proteins are known to play a role in maintaining P homeostasis in *Arabidopsis* during Pi starvation and are involved in the release of Pi from phospholipids as a response to Pi starvation. AtGDPD1 loss of function *Arabidopsis* seedlings has been documented to result in lower Pi content and plant growth compared to the wild type seedlings during Pi starvation [Cheng et al., 2011], rice has also displayed that GDPD activity leads to lipid remodelling and improved low Pi tolerance in response to Pi deficiency, with GDPD activity resulting in increased Pi content in rice [Mehra et al., 2019].

Multiple proteins relating to lipid remodeling were identified in various *B. rapa* R500 proteomes, these proteins identified and the samples they were identified in are represented in Table 7.3, proteins identified in Col-0 exudates in response to Pi starvation are also included using the *B. rapa* homologue of the identified Col-0 proteins. GDPD proteins were seen in multiple samples to be increased in abundance in response to Pi starvation, they were identified in the *Arabidopsis* and *B. rapa* exudates, showing a similarity between the species response to Pi starvation regarding lipid remodelling. Three BrGDPD proteins were identified in the *B. rapa* root proteome to increase in abundance. And four had increased expression and one decreased expression in the *B. rapa* shoot transcripts. There was also an significant enrichment in GO term molecular functions for GDPD activity in the root membrane and root soluble proteome of Pi starved *B. rapa*. This increase in GDPD proteins during Pi starvation indicate that *Arabidopsis* and *B. rapa* roots undergo lipid remodelling to improve phosphate utilisation efficiency as a PSR.

Other proteins involved in lipid remodelling that increase during Pi starvation include some lipase proteins, *B. rapa* phospholipase A1 and A2 is increased R500 shoot transcripts and roots proteome respectively. The *Arabidopsis* phospholipase A1 homologue has been documented previously to deesterify membrane phospholipids but have not been documented to be Pi starvation induced [Lo et al., 2004]. Proteins involved in SQDG synthesis increased in both the roots and shoots *B. rapa* proteomes (Table. 7.3). Two MGDG synthase genes (BrMGDG2/3) also increased in expression in response to Pi starvation in R500 shoots but the proteins were not detected. The MGDG synthase 2 gene expressed is a homologue to AtMGDG synthase 2 which has been shown to have increased expression during Pi starvation and is linked with lipid composition [Kobayashi K et al. [2006]].

The AtDGDG synthase 2 (Q8W1S1), phosphorylated at site S466 increased in abundance in response to Pi starvation in the Col-0 phosphoproteome, AtDGDG synthase 2 is involved in the cellular response to Pi starvation biological process GO term. The same modification has been reported on PhosPhAt in *Arabidopsis* seedlings in response to drought stress [Bhaskara et al., 2017], but neither the protein or the phosphorylation has been reported as responsive to Pi stress previously in *Arabidopsis*, but it does fit in with the lipid remodelling pathway PSR. However, the role phosphorylation may have on the proteins function is unclear. Another protein identified in Col-0 phosphoproteome to have increased phosphorylation detected in response to Pi starvation which is a part of lipid remodelling pathway is Phosphatidate phosphatase 1 (Q9SF47), with the phosphorylation detected at S162, Phosphatidate phosphatase 1 protein has been shown previously to play a role in membrane lipid remodelling in response to Pi stress [Nakamura et al., 2009], but the phospho-modification, while it has been documented before [Wang et al., 2013], has not been documented in response to Pi stress.

The number of proteins and genes that are responsive to Pi starvation that are involved in the lipid remodelling pathway,indicate that lipid remodelling is a PSR shared between species and multiple tissue types. This pathway is a good candidate for future work to pinpoint specific proteins and functions that play a role in different plants to improve Pi recycling and efficiency.

Table 7.3: Comparison of lipid remodelling proteins identified in the different *B. rapa* proteomes and Col-0 exudates in response to Pi starvation: The proteins which had significantly changed in response to Pi starvation related to lipid metabolism are are indicated with arrows in what *B. rapa* sample they were identified, the corresponding *B. rapa* homologues identified in the Col-0 exudates are also represented.

Protein ID	Protein Name	Col-0 Exudates	Exudates	Root	Shoot	Shoot Transcripts
A0A3P5ZTK7	GDPD3	↑	↑	↑		
A0A3P6CG48	GDPD4	↑	↑			
A0A3P6A2W2	GDPD3		↑	↑		
A0A3P6C0P5	GDPD5			↑		
A0A3P6B1U2	GDPDL3					↑
A0A397Y582	GDPD3					↑
A0A397XSY4	GDPD6					↑
A0A3P6B1T5	GDPD2					↑
A0A398AH49	GDPDL1					↓
A0A3P6A0K3	MGDG synthase 2					↑
A0A3P5YCT3	MGDG synthase 3					↑
A0A397ZA59	Phospholipase A1					↑
A0A397ZQK6	Phospholipase A2			↑		
A0A3P6A432	UDP-sulfoquinovose synthase				↑	↑

Continued on next page

Table 7.3 – *Continued from previous page*

Protein ID	Protein Name	Col-0 Exudates	Exudates	Root	Shoot	Shoot Transcripts
A0A3P5ZE00	SQD2			↑		↑

7.5 PEPC proteins get phosphorylated in response to phosphate starvation

PEPC proteins play a role in maintaining Pi homeostasis in response to Pi stress by offering an alternate metabolic route during low Pi availability in *Arabidopsis*. AtPEPC1 phosphorylation of S11 is reversibly phosphorylated and has previously been documented to occur in *Arabidopsis* in response to Pi deprivation, resulting in an increased activity of the protein, PEPC is targeted by PEPC kinase, and the metabolic bypass provided allows improved Pi efficiency [Gregory et al., 2009, Plaxton and Shane, 2015].

As discussed earlier *Arabidopsis* Col-0 phosphoproteome and *B. rapa* phosphoproteomes doth displayed an increased PEPC phosphorylation at S11. Table 7.4 displays what PEPC and PEPCK proteins and phosphomodifications were identified to change in abundance in response to Pi starvation.

AtPEPC1 and AtPEPC3 are phosphorylated at S11 in Pi starved conditions, while a AtPEPCK2 is shown with decreased phosphorylation during Pi starvation. The shoots of *B. rapa* also have four BrPEPC proteins which increased in phosphorylation abundance at S11, and the *B. rapa* roots also have two of these BrPEPC phosphopeptides increased in response to Pi starvation. BrPEPC proteins were also changed in abundance in the *B. rapa* proteome samples not just the phosphoproteome samples, the shoots have an increase in abundance and expression of BrPEPC1 in response to Pi stress while there is a decrease in BrPEPC2 expression, while also having a decreased abundance of BrPEPCK1. The increased abundance of BrPEPC in both the proteomes and phosphoproteomes means we cant determine for certain whether the phosphorylation of the BrPEPC changes in response to Pi or if it is just the overall abundance of the protein that changes. The abundance of PEPCK does not always correlate with the increased phosphorylation detected on PEPC proteins, but we cant say what PEPC proteins are targeted by what kinase and there may be other proteins involved in this pathway that have not been identified.

The various responses of different PEPC and PEPCK proteins and their phosphorylation in response to Pi availability indicate they play an role in *B rapa* PSR, which has not been documented before. But the specific roles of each proteins and what proteins are targeted by what kinase, and the effects of the pathways will need further research to piece together.

Table 7.4: Comparison of PEPC proteins identified in *B. rapa* in response to Pi starvation: The proteins that significantly changed in response to Pi starvation are indicated with what samples they were identified in, and the phosphomodification identified to change in abundance is also indicated. Including the *B. rapa* homologue of the *Arabidopsis* PEPC proteins identified in the Col-0 exudates.

Protein ID	Protein Name	Root	Shoot	Transcripts	Root phospho-proteome	Shoot phospho-proteome	Col-0 phospho-proteome
A0A397Y6V5	PEPC1	↑	↑	↑		↑ S11	↑ S11
A0A3P6CTA5	PEPC2		↓			↑ S11	
A0A3P5YR48	PEPC2	↑		↑	↑ S11	↑ S11	
A0A3P5Z9C2	PEPC3				↑ S11	↑ S11	↑ S11
A0A3P5ZNL1	PEPC	↑					
A0A3P5Z0Z4	PEPCK 1	↑	↓				
A0A3P5Z150	PEPCK 2	↑		↓			↓ S66
A0A3P5YCU5	PEPCKL 1			↑			
A0A398A8H9	PEPC3			↑			

7.6 RNase proteins are responsive to phosphate levels

Another protein group that was detected in a various samples to respond to Pi starvation were RNase proteins. Nucleic acids are a rich source of Pi that can be scavenged and recycled in times of low Pi availability. Induction of RNase proteins has been documented in tomato plants in response the Pi stress to play a role in scavenging phosphate from extracellular RNA sources [Abel et al., 2000].

The RNase proteins identified to respond to Pi starvation in different *B. rapa* samples are displayed in Table 7.5, and indicate whether they are increased or decreased in abundance in response to Pi starvation.

B. rapa exudates had three Ribonuclease T(2) proteins increased in abundance in response to Pi starvation. The ribonuclease T(2) 1 *Arabidopsis* homologue (P42813) was also identified to have increased in Pi starved Col-0 exudate proteome. Another Ribonuclease T(2) *Arabidopsis* homologue (Q9XI64) was also detected in Col-0 exudates but was not determined to be significantly changed in abundance. Two of the *B. rapa* ribonuclease T(2) proteins were also increased in abundance in the *B. rapa* roots in response to Pi starvation. These ribonuclease proteins were not identified in the shoot proteome but were in the shoot transcripts.

Ribonuclease T(2) activity is an enriched GO term molecular function seen in Pi starved *B. rapa* exudates and root proteins indicating a significant enrichment of these proteins seen in response to Pi starvation. They are potential candidats for

improving *B. rapa* Pi efficiency by improving the accessibility of Pi from RNA sources.

The *B. rapa* root proteome also identified two ribonuclease TUDOR proteins which decreased in abundance in response to Pi starvation, the shoots also had one which decreased in abundance, these are homologues to the *Arabidopsis* ribonuclease TUDOR 1 and 2 proteins. Ribonuclease TUDOR1 protein in *Arabidopsis* has been shown to play a role in abiotic stresses like cold and salt stress, by regulating the metabolism of mRNA involved in secretory pathways [dit Frey et al., 2010], but has not been previously documented to be Pi responsive.

Table 7.5: Comparison of RNase proteins identified in *B. rapa* in response to Pi starvation: The proteins that significantly changed in response to Pi starvation are indicated with what samples they were identified in. Includes the *B. rapa* homologue of the *Arabidopsis* RNase proteins identified in the Col-0 exudates.

Protein ID	Protein Name	Exudates	Root	Shoot	Transcripts	Col-0 exudates
A0A3P6AUK7	Ribonuclease T(2) 1	↑	↑		↑	↑
A0A397YEW7	Ribonuclease T(2)	↑	↑		↑	
A0A397Z3E6	Ribonuclease T(2) 1	↑				
A0A3P6A2G7	Ribonuclease TUDOR 1		↓			
A0A3P5YHS7	Ribonuclease TUDOR 2		↓	↓	↑	

7.7 Sucrose metabolic proteins change in response to phosphate availability

Sucrose signalling plays a role in a plants response to Pi starvation, transporting in the phloem leading to shoot to root communications acting as a signal to co-ordinate root responses to Pi deficiency, with an increase in sugar concentration within the phloem documented to occur before a plants PSR is initiated [Hammond and White, 2008].

The GO term enrichment analysis in my data also suggests an increase in carbohydrate biosynthesis genes expression during the *B. rapa* PSR. The increased gene expression in *B. rapa* shoots during Pi starvation has an enrichment in cellular carbohydrate metabolic process, there was also an increased abundance of sucrose-phosphate synthase (SPS) proteins identified in the *B. rapa* shoot proteome, correlating with their increased gene expression. There was also an increase in BrSPS phosphorylation identified in Pi starved *B. rapa* shoots, with three BrSPS homologues; two BrSPS1 and BrSPS4 (AOA397XKW7, AOA3P6A8T1, AOA3P6AP98), having an in increased phosphorylation identified at residues S696, S714, and S699

respectively. These phospho-modifications have not been identified in *B. rapa* previously to the best of my knowledge. The *Arabidopsis* homologue AtSPS1 has been identified to have multiple phosphorylation sites, one of which in the corresponding S700 residue active in response to flg22 treatment according to PhosPhAt [Rayapuram et al., 2018], but the phosphorylation has not been documented in response to Pi deprivation previously, and any potential function of the phosphorylation on the protein is unknown. The *Arabidopsis* AtSPS4 homologue, has not documented a phospho-modification identified in the corresponding position identified on the *B. rapa* BrSPS4 homologue, and has not previously been documented to play a role in Pi efficiency, but is suspected to have a role in sucrose availability in *Arabidopsis* [Sun et al., 2011]. These proteins identified in *B. rapa* could suggest candidates involved in a PSR relating to sucrose signalling and increased biosynthesis, but future research on their functions and whether they are involved with increasing sucrose concentrations and signalling would be required.

7.8 Peroxidase proteins were consistently observed to change in abundance during Pi starvation in *B. rapa*

Peroxidase proteins play a role in the detoxification of reactive oxygen species during stress responses, they reduce peroxide through substrate oxidation therefore alleviating oxidative stress. Peroxidase proteins are a large family of proteins found in a variety of organisms. The plant peroxidase superfamily is split into three classes [Welinder, 1992]. Class 1, called ascorbate peroxidase because their main electron donor is ascorbate, are found in plants and bacteria. Class 2 are fungal extracellular peroxidases, and Class 3 is the largest class and is called the plant peroxidases, they are commonly secreted or transported into vacuoles [Hiraga et al., 2001, Lazzarotto et al., 2015].

Peroxidase proteins were identified in various *B. rapa* proteomes, but their response to Pi starvation was varied. *B. rapa* exudate proteome displayed five peroxidase proteins increased in abundance during Pi starvation, in contrast to the *Arabidopsis* Col-0 exudate proteome, which identified three decreased in abundance and one increased in abundance. *B. rapa* membrane associated root proteome identified nineteen peroxidases increased in abundance during Pi starvation. While the root and shoot soluble proteomes displayed an even split of peroxidase proteins both increasing and decreasing in abundance. The change in peroxidase abundance is

also indicated with the enrichment of peroxidase activity molecular function GO term, showing that there is a significant response of peroxidase proteins in response to Pi starvation, but the exact role the different proteins play in different samples is unknown.

There could be a specific role that some of the peroxidase proteins play in response to Pi starvation, or it could be a more general stress response to there being nutrient deprivation, they could also be a stress response to the general growth and harvesting of the plants during the experimental procedure. A Wound response will lead to increased peroxidase activity leading to ROS (reactive oxygen species) burst, and further signalling to lead to would healing [Minibayeva et al., 2015]. Some peroxidase proteins are particularly effective at producing ROS, while others are more sufficient at the removal of ROS [Minibayeva et al., 2015]. This could explain why there are some peroxidases which increased and some which decreased in abundance in response to Pi starvation. Further research would be needed to determine the different functions that different peroxidase proteins could have during Pi starvation.

7.9 Future Work

I have shown several key changes in *B. rapa* proteomes in response to Pi starvation. Some of these changes could be responsible for the improvement of Pi uptake and utilisation efficiency to better help the plants adapt to the low Pi conditions. Unfortunately this work is primarily observational changes and does not include the functionality of the proteins, just the abundance and presence of them, therefore I propose that the most useful future work that could be continued following these results would be functional characterisation of some of the proteins or protein groups observed to most consistently or significantly change during a PSR. Time permitting generating knockout and overexpression lines of proteins of interest would have provided valuable insights into the functionality of the proteins to the plant, but was not possible in the time frame of the project, partly due to the difficulty and time requirement for generating these lines in *B. rapa* plants.

The PAP family of proteins for example would have been a good target for protein characterisation. Some previous studies have been done in *Arabidopsis* and rice to see if the overexpression of certain PAPs can improve Pi uptake. Overexpression of AtPAP10 [Wang et al., 2011a] and OsPAP10 [Tian et al., 2012] led to increased growth during low Pi conditions. Continuing along this thread in *Brassica* I think

could prove most fruitful to improve crop growth during low Pi availability. As mentioned in the introduction, the agricultural community can not rely on the use of Pi fertilisers to improve crop yield when they are a finite nonrenewable resource, therefore improving on the crops capability to improve their own growth in during Pi availability would be in the long term more sustainable and beneficial to improving crop yields during Pi deficiency.

Cloning and characterising the individual PAP proteins would be useful to determine the functions and activity of the proteins. Due to the similarity within the proteins it is often difficult to confirm exactly which PAPs might have been identified within the proteomes. It was also not possible to confirm which PAPs could be responsible for the changes in APase activity observed with the activity assays, therefore characterising the individual PAP proteins might have helped to pinpoint which PAPs were involved in increasing function, as well as determining substrate specificity, and not just showing an increase in abundance. This work was underway for the BrPAP1, 10 and 15 proteins which I identified to increase in abundance in response to Pi starvation in *B. rapa*, but unfortunately time ran out before I could successfully develop activity assays for functional characterisation.

Other work which I believe would be useful to continue will be the identification and analysis of other exudates secreted from the roots of Pi starved *B. rapa*. The secretion of organic acids and sugars plays a role in Pi utilisation for various plant species, such as *Arabidopsis*, tomato and lupin [Dixon et al., 2020, Pantigoso et al., 2020, Wu et al., 2018], but the secretion of organic acids in *B. rapa* in response to Pi levels is not yet as well documented and could provide insight into the other roles of secretion for improved Pi uptake efficiency in *B. rapa*. The secretion of organic acids by plant roots is expected to play a role in a variety of processes including improved Pi acquisition as well as the recruitment of beneficial rhizosphere bacteria [Wu et al., 2018]. Organic acids have a chelating function to help solubilise Po within rhizosphere to better able them to be accessible to the plants, this can be achieved by the secretion of, for example malate, fumarate and oxalate [Bolan et al., 1994, Dixon et al., 2020, Maruyama and Wasaki, 2017]. Utilising selective reaction monitoring MS would allow the identification of root excretions from growth media to compare Pi starved plants to control plants to see how the secretions change. It is a specific technique that will selectively quantify compounds, so we would need to know what to look for, thankfully previous studies in other plant species already identified potential candidates for what compounds would be useful to look for, then knowing what changes in response to Pi deprivation can indicate what organic acids

get secreted and play a role to improve Pi acquisition.

Transporter proteins have also shown some promise in overexpression lines at improving Pi uptake even when in low Pi conditions in a variety of species, for example high affinity *Arabidopsis* PHT1 in tobacco cultures [Mitsukawa et al., 1997] and the rice PHT1;3 transporter [Chang et al., 2019]. Therefore overexpression *B. rapa* high affinity Pi transporters could help to improve Pi uptake during low Pi conditions. However this runs the risk of toxicity in the plant in the event that there is ample Pi availability, but too much Pi uptake, causing Pi toxicity.

There are a wide variety of directions that further research could be taken to improve *B. rapa* Pi efficiency. Utilising the proteins that have been identified to be responsive to Pi starvation could be used to further improve the plants Pi efficiency in low Pi conditions.

7.10 Conclusions

The overarching conclusion of this research is that there is a drastic and significant change in *B. rapa* and *Arabidopsis* plants proteome, transcriptome, and phosphoproteome in response to Pi starvation. While a number of the proteins that are Pi responsive are similar between species there are also a number of new Pi responsive candidates that have been identified in *B. rapa*, including PAP proteins, lipid remodelling proteins, and RNase proteins. A number of potential signalling components have also been identified, many of which are phosphorylated which have not been identified previously, particularly in *B. rapa*, with cases where the phospho modifications have not been recorded in the *Arabidopsis* homologues. The identification of these protein changes in *B. rapa* in response to Pi starvation is interesting and new, but needs to be followed up with more functional experimentation to get a better idea of how these changes in protein abundance really affect the plants Pi efficiency, and enable us to utilise them to further enhance *B. rapa* Pi efficiency.

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